HISTOLOGICAL TECHNIQUE

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A GUIDE FOR USE IN A LABORATORY COURSE IN HISTOLOGY

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PREFACE

The following represents the combination of technique notes written by the first author for use in connection with courses in Histology offered by him for medical, premedical, and veterinary students, with a similar outline of histological methods designed by the second author for use in courses dealing primarily with the histology of insects.

Inasmuch as the methods for the microscopic examination of animal structure are fundamentally the same, whether the structure is normal or pathological, the approach medical or zoölogical, it is believed that there has been here produced a book of much broader usefulness, without in any way sacrificing its value in histological work of more specific application.

A rigid selection has been exercised, so that of the multitudinous methods employed in microscopic work only those are here given which meet the requirements for attaining a broad practical knowledge of animal structure. In special investigations it is necessary to make a study of the particular technical needs of the problem, and for this it is well to consult the larger works, of which may be mentioned: The Encyclopedia of Microscopic Technique [10]; The Microtomist's Vade-mecum, by A. B. Lee [35]; Mallory and Wright, Pathological Technique [38]; Schuberg, A. [46]. These, as well as other books and articles, are listed in the Appendix, and reference is made to them in the text, either directly or by number [in brackets]. Furthermore, in many instances, direct reference to important papers is given in the text, thereby increasing the usefulness of the book for advanced students in special fields.

While the aim has been to present methods for the microscopic examination of any animal form, the emphasis is nevertheless placed on the technical needs of the premedical (or medical) student and the student of Entomology.

For the loan of most of the cuts the writers are indebted to the Bausch Lomb Optical Co., E. Leitz, Inc., Spencer Lens Co., the Will Corporation, and C. Zeiss, Inc.

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INTRODUCTION

Very few structures of the animal organism can be adequately examined microscopically without being first subjected to a preparatory treatment involving in many cases the employment of complicated methods. Save in the case of the body fluids and certain membranes, animal tissues are bulky, more or less opaque, and therefore unsuited for examination under the microscope, which requires surface or thin layers of substance. Examination is made possible in such cases in one of two ways: the elements composing the structure may be separated from each other, or thin slices may be prepared.

The above, however, presents but the grosser aspect of the necessity of preparation of animal tissues for examination with the microscope. The histological analysis of bodily structure makes further demands on the refinement of methods. Treatment with chemicals and stains (fixation and staining) has for its purpose not only the preservation and delineation of structure, but also its identification by means of more or less definite chemical (physical) reactions. The goal, from this side of histological technique, is an analysis from the chemico-physical as well as the morphological aspect and the interpretation of morphology in terms of physiology. Increase in our knowledge of the finer structure of the body in the past has been, as advance in the future will be, accompanied by and dependent on the application of a more exact technique along these lines; while for those who aim to do practical work in histology and pathology a mastery of the more important methods is indispensable.

Furthermore, in working with chemically altered structure, there is always the danger of losing sight of the conditions existent in the living protoplasm. It is well, therefore, in addition, to study structure in the living or fresh state, as little altered from the natural as may be. It is also very desirable to acquire skill in the application of simple methods which require neither expensive apparatus nor expenditure of time, — methods which, while they may not advance knowledge, serve often to meet the needs of a preliminary examination or rapid clinical diagnosis.

HISTOLOGICAL TECHNIQUE

FIXATION

1. Fixation is one of the fundamental processes in the examination of plant and animal tissues. A *fixer* may be defined as a fluid (or gas) in which the living, or at least very fresh, tissue is placed in order to preserve the structure of its elements as nearly as possible as in life. Living tissue, when allowed to die and remain undisturbed, gradually loses the structural features it had in life and undergoes disintegration and decay. Fixation depends upon physico-chemical processes wherein the chemical constituents of the tissue are thrown down *in situ* by being rendered insoluble in some form or represented by substitution products, the whole being attended by as little distortion as possible. It should be appreciated that the chemical constituents of cell protoplasm and of the tissues are numerous and diverse in their chemical and physical properties, so that a universal or ideal fixer not only does not exist but is logically inconceivable. The bulk of protoplasm and the tissues is *protein*, and the basis of fixation in general is the precipitation or coagulation of these chemical substances. It should be remembered, however, that fats (lipoids[1]) are a constant though variable component of cytoplasm; that carbohydrates (glucose, glycogen, etc.) are usually present in small amounts; and that the products of cell activity, such as secretion granules, zymogen, etc., may be quite distinctive in their physico-chemical properties. Within the cell a certain "antagonism" exists between nucleus and cytoplasm, the former seemingly oxidative, the latter reducing, requiring often somewhat different fixation conditions.

For the best results, the fixer should be chosen with a view to the preservation of some particular part or constituent, though a

[1] Lipoid, while not a good chemical term, is one that is quite useful in histology, to include fats, fatty acids, phosphatids, cholesterol, etc., substances that have the same solvents and which are found associated in protoplasm.

number of *general fixers* are very serviceable for routine work. *Rational fixation* will depend upon a detailed knowledge of the chemical and physical properties of the constituents that it is desired to preserve and their interaction with the chemicals of the fixer. In many respects, rational fixation still awaits further knowledge of the physics and chemistry of fixation.

2. The chemicals of most service as fixers are: (1) osmic acid (osmium tetroxid), (2) platinic chlorid, (3) picric acid (trinitrophenol), (4) acetic acid, (5) chromic acid, (6) mercuric chlorid, (7) alcohol (ethyl or methyl), (8) potassium dichromate, (9) copper dichromate, (10) formaldehyde (formalin, formol), (11) nitric acid. These are best used in combinations in which their actions are mutually supplemented or their defects corrected.

3. Coagulation and Precipitation. As the chemical basis of protoplasm and the animal tissues is *protein*, the most important characteristic of fixation is rendering these insoluble by *coagulation*. Platinic chlorid, mercuric chlorid, and chromic acid are most intense and comprehensive in their coagulative action. Picric acid is a delicate *precipitant* of proteins, but the resulting precipitate is soluble in water, hence the rule to wash out these fixers (*vide subseq.*) in alcohol (67–82 per cent). Acetic acid precipitates particularly the nuclear proteins (nucleins, nucleo-proteins). As the precipitation of proteins proceeds better in the presence of acid, general fixers should be acid in reaction ($\frac{1}{10}$ to 5 per cent acetic acid, etc.). Osmic acid, potassium dichromate and certain other dichromates, and formalin do not seem to coagulate proteins. They are poor fixers of nuclear structures but are good cytoplasmic fixers and preservers of lipoids (§ 240).

4. Penetration. This is necessarily an important requisite in a fixer. Nitric acid, trichloracetic acid, acetic acid, potassium dichromate, and formalin possess (in order given) good penetrative power. Platinic chlorid, picric acid, osmic acid, and chromic acid penetrate but poorly. Mercuric chlorid diffuses but fairly well. Concentration in the solutions and increase in temperature facilitate penetration as well as coagulation, hence, in general, strong solutions are used. In some cases fixation at incubator (body) temperature (35–38° C.) is to be advised, while with very impervious objects fixation in boiling-hot fixer is advisable.

Fixation by Injection. To facilitate speedy penetration, it is frequently advisable to inject the fixer, ordinarily through the artery supplying the part. Physiological salt solution (§ 151) is first injected to remove the blood, and is followed by the fixing fluid.

5. Mechanical Distortion. Osmic acid, potassium dichromate, and formalin seem to possess a tendency to cause swelling, while chromic acid and alcohol make the tissue shrink.

6. The fixing solutions (§ 11) mentioned below, which contain these salts, usually in combination, are useful: (1) mercuric

chlorid and acetic acid; (2) Zenker's fluid (dichromate, sublimate-acetic), (3) Zenker-formol; (4) formol-dichromate (Orth's fluid); (5) copper dichromate-sublimate-acetic; (6) picro-aceto-formol; (7) osmo-aceto-dichromate (Bensley's); (8) Hermann's fluid (platino-aceto-osmic); (9) Flemming's fluid (chromo-aceto-osmic); (10) Champy's fluid; (11) Carnoy and LeBrun's fluid; (12) Gilson's fluid (mercuro-nitric); (13) Petrunkevitch's fluid; (14) picro-nitric; (15) picro-aceto-sublimate (v. Rath's). The following, while not balanced fixers, are serviceable as general fixing fluids or for special purposes: (16) alcohols; (17) alcohol-acetic; (18) chloroform-alcohol-acetic; (19) alcohol-acetic-formol; (20) formaldehyde (formalin); (21) osmic acid (osmium tetroxid); (22) Dietrich's fluid; (23) Van Leeuwen's fluid. Obsolete as fixers but having a usefulness in certain cases, particularly in the technique of the central nervous system (§§ 213, 216, 218), are: (24) Müller's fluid; (25) Erlicki's fluid; (26) potassium dichromate (in aqueous solution).

In the combination of chemicals in fixing solutions such as those mentioned above, they should be chosen to supplement each other's actions as far as possible and correct or counteract each other's defects. The combinations must be chosen always with a view to the result desired, and frequently the components and their relative amounts must be determined empirically, — by experiment.

7. The following general rules should be regarded in the fixation of tissues and organs:

(1) The volume of the fixing fluid used should be large, exceeding the volume of the tissue at least thirty times. The less energetic the action of the fixer the greater the amount of fluid to be employed. When the fluid becomes turbid it should be changed to fresh at once.

(2) Fix only as small pieces of tissue as possible, or as is practicable in view of the results desired. The block of tissue should not be more than 1 cm. in one dimension, and if possible should be much shorter, — only 2 to 5 mm. In some cases (Flemming's and Hermann's fluids) much smaller masses are needed (1 to 2 mm. thick). This is desirable for the rapid and complete penetration of the fixer. Of course, in the case of entire organs it may not be possible to comply with the conditions. Fixation by injection may then be resorted to. Physiological salt solution (§ 151) is first

injected through the artery of the organ or part in order to remove the blood, and this is followed by the injection of the fixing fluid.

In addition to these two general principles, there are four points to be carefully considered, upon which the excellence in the results attained depends; they are (*a*) the *fixer* chosen, (*b*) the *time* of fixation, (*c*) the *washing out* of the fixer, and (*d*) the *hardening* in alcohol and the subsequent treatment.

(*a*) The choice of the fluid in which the tissue is placed should depend on (1) a consideration of the particular feature whose preservation is desired and the degree of excellence of fixation that is desired or necessary, — whether detail of cell structure or the structure of the tissue in terms of cells or structural elements be sought; (2) the penetrating power and the size of the piece that it is necessary to have; and (3) the stain that is desired subsequently, which is largely determined by the fixation.[1]

(*b*) The time a fixer is allowed to act should be considered in connection with the character of the fluid and the tissue. Usually the exact limitation of time is a matter of secondary importance and the tissue may remain in the fixer indefinitely. In some cases, however, disregard of the time of fixation affects the results seriously, and as a general rule there is a minimum and a maximum time and, between them, an optimum time that should be adhered to. However, it is only in the case of chemicals that act progressively, such as strong acids and oxidizers (e.g., osmium tetroxid, potassium dichromate, etc.), that over-fixation must be avoided.

(*c*) After the tissue has been in the fixing fluid a proper length of time, it must be washed thoroughly to remove the fixer from it. Usually this should be done by means of water or alcohol, or both. In general, fixers containing salts insoluble in alcohol or but slightly soluble, as osmic acid, chromic acid, potassium dichromate, etc., should be thoroughly washed in water. Fixers containing picric acid or alcohol should always be removed by alcohol; mercuric chlorid may be washed out by either water or alcohol.

Inadequate washing out of the fixer may either seriously affect the cutting quality of the tissue (if it is to be subsequently im-

[1] Numerous papers have been written on the nature of fixation and the action and relative value of the various chemicals used for that purpose. The contributions of Berg [3], Fischer [11], Mann [39], and v. Tellyesniczky [10] may be particularly mentioned.

bedded) or the ease with which it can be stained, or may cause precipitates to be formed in the tissue, giving illusory effects, distortions, or at least a dirty appearance to the preparation. Time is always well spent in properly washing out a fixer, as it is a matter for serious attention.

8. Transferring Objects from One Fluid to Another. When a delicate object is transferred from a fluid to one differing greatly in density, more or less violent diffusion currents are set up which may cause serious swelling, shrinking, or distortion. To avoid this, various expedients are adopted. The smaller and the more delicate the object the greater the importance of meeting this technical difficulty; it is therefore primarily in cytological work that it requires attention. In ordinary cases it suffices to pass the material through several grades of a mixture of the two fluids. Thus, in transferring from weak alcohol to absolute, several grades of intermediate strength are interpolated; or in passing from absolute alcohol to cedar oil, objects may first be transferred to a mixture containing $\frac{3}{4}$ alcohol and $\frac{1}{4}$ cedar oil, then to a mixture of equal parts of each, then to $\frac{1}{4}$ alcohol and $\frac{3}{4}$ cedar oil, before passing to the pure oil. Very small objects may be placed in short lengths of glass tubing with a pierced cork at one end and a gelatin-coated gauze at the other. See also Metz (Anat. Rec. 21 : 373, 1921). When the two fluids mix slowly, as with cedar oil and alcohol, the two may be superimposed in a slender shell vial, the oil below, the mixture in between, and the alcohol on top. The object in the alcohol will slowly sink to the bottom, thus allowing time for a gradual replacement of the alcohol.

When the transition must be very gradual, as in certain cytological work, the alcohol series may be 2.5, 5, 7.5, 10, 15, 20, 30, 40, 50, 70, 85, 95, 100 per cent, the small pieces of tissues being allowed to remain 1 to 2 hours in the lower grades and a little longer in those over 50 per cent; or the second fluid may be added drop by drop with a pipette. If the quantity of fluid is considerable this is very time-consuming, and therefore various devices have been described. For these the reader is referred to various papers dealing with the so-called drop method. Allen (Anat. Rec. 10 : 565, 1916); Bishop (Trans. Amer. Micr. Soc. 36 : 1917); Guyer (Animal Micrology 1917); Hance (Trans. Amer. Micr. Soc. 1925); Long (Anat. Rec. 29 : 324, 1925); Painter (Anat. Rec. 27 : 1924).

HARDENING AND STORING

9. Each fixer has also more or less of a hardening action upon the tissue. Some fluids spoken of above as fixers, such as Müller's fluid and Erlicki's fluid, were primarily used as hardeners, while with others, e.g., picric acid in aqueous solution, the hardening action is a minimum. The hardening action of the fixer is generally supplemented by the subsequent use of alcohols of increasing strengths (50 per cent to absolute, — 99 per cent), as well as in preparation for the paraffin and celloidin methods of imbedding. In fact, with modern methods of imbedding, excessive hardening of the tissue is not neces-

sary and indeed often should be avoided as affecting the cutting quality of the tissue. Tissue, after fixation has been completed, may be stored in 82 or 95 per cent alcohol, or (better) imbedded at once (§ 48).

Alcohols. Fifty, 70, and 80 or 85 per cent alcohols form a series sufficient for most purposes. Approximations (48, 67, 82 per cent) are close enough for ordinary work and may be easily prepared from 95 per cent alcohol by taking (*a*) for 50 per cent alcohol, 95 per cent alcohol 1 part, water 1 part; (*b*) for 67 per cent alcohol, 95 per cent alcohol 2 parts, water 1 part; (*c*) for 82 per cent alcohol, 95 per cent alcohol 5 parts, water 1 part. Dilutions of other strengths may easily be prepared as desired from 95 per cent alcohol. Ninety-five per cent (94 per cent) alcohol and absolute alcohol are necessary in imbedding by the paraffin and celloidin methods (§ 61).

To Obtain Different Dilutions of Alcohol. From the commercial grain alcohol of 95 per cent, the lower percentages called for in the directions may readily be obtained by dilution with distilled water in accordance with the following method. Pour into a 100-cc. graduate as many cubic centimeters of the 95 per cent alcohol as the amount of the percentage required, and then add water until the mixture reaches the 95-cc. mark. For example, if a 70 per cent alcohol is required, pour into the graduate 70 cc. of the 95 per cent alcohol and then add 25 cc. of distilled water. The result is 95 cc. of 70 per cent alcohol. The same method may, of course, be used to obtain a lower percentage from one of any higher percentage. Thus, to obtain a 60 per cent from an 80 per cent alcohol, add to 60 cc. of the 80 per cent, 20 cc. of water, making 80 cc. of 80 per cent alcohol.

10. Stock Solutions. It is advantageous to have on hand strong solutions of the chemicals employed as fixers and stains. Where feasible, 10 per cent solutions are most convenient. The following are the more important: In aqueous solution, 10 per cent potassium dichromate, 10 per cent copper dichromate, 10 per cent chromic acid, 10 per cent platinic chlorid, 40 per cent formaldehyde (formalin), 4 per cent sodium sulphate, 4 per cent copper sulphate, 2 per cent osmic acid, saturated solution of mercuric chlorid, saturated solution of picric acid, 95 per cent alcohol, absolute alcohol, etc., as well as the strong acids, stock staining solutions, etc.

FIXERS

11. Mercuric Chlorid. Fluids containing mercuric chlorid should not be allowed to come in contact with metal implements as it is exceedingly corrosive. One may employ a saturated solution in water with 1 to 5 per cent glacial acetic acid. Water will dissolve about 5 per cent of the mercuric chlorid. This is a good fixer, especially when the piece is small. It fixes as soon as it penetrates and is apt to make tissue brittle if it is left too long. Staining after it is brilliant. The larger percentage of acetic acid is, perhaps, to be preferred for most histological objects.

Fix the fresh tissue for ½–24 hours, according to the size of the piece. Remove to 67 per cent alcohol for 1 or 2 days, 82 per cent alcohol several days, changing often. The 82 per cent alcohol should contain enough tincture of

(6) *Cedar oil.* A most useful clearer for most structures as it does not render them brittle and seems to improve the cutting quality. It is preferably employed only with small objects, and the paraffin should be changed at least once after it. It will, if necessary, clear from 95 per cent alcohol, but the presence of water will cloud the molten paraffin, making it difficult to find small objects. A small object placed in xylene (toluene, benzene) for a short time, after having been cleared in cedar oil, is easier to handle in the paraffin. This is sometimes also advisable for large objects that have been cleared either in cedar oil or in carbol-xylene.

(7) *Clove oil.* This clearer is also a solvent of celloidin (§ 138). It is recommended for chitinous structures and for minute dissections before the mounting.

(8) *Methyl benzoate.* A clearer and a better solvent of celloidin. It does not darken with age as does clove oil. Its odor may be objectionable to some.

(9) *Turpineol.* This may be used as a clearing agent instead of clove oil, but it does not dissolve celloidin.

(10) *Essential oils.* A number of these may be employed. There may be mentioned oil of wintergreen (methyl salicylate), oil of bergamot, oil of origanum, oil of thyme.

(11) *General solvents.* A number of these make excellent clearers; and since they mix with alcohol often of low percentages, even with water, and also dissolve paraffin, they may thus be employed for both dehydration and for clearing. There may be mentioned: *Acetone; Amyl acetate; Butyl alcohol* (butanol); *Butyl acetate; Butyl aldehyde; Diethyl dioxid* (Dioxan); *Methyl benzoate* (No. 8 above); *Propyl alcohol.* The list might be extended.

All of the above dissolve Canada balsam (§ 162). All save dioxan will dissolve celloidin (nitrocellulose) (§ 61). All will mix with melted paraffin, but only dioxan and methyl benzoate dissolve it readily. All mix with 95 per cent alcohol but only acetone, dioxan and propyl alcohol mix readily with water. For the use of amyl acetate, see Barron, Anatomical Record, vol. 59, p. 1. For Dioxan, see Turtox News, vol. 12, No. 5, p. 162.

The following succession of steps will indicate the procedure to be followed in clearing tissue for paraffin infiltration:

	Toluene (xylene, benzene)	Chloroform	Cedar Oil
1.	Absolute alcohol.	Ninety-five per cent or absolute alcohol.	Ninety-five per cent or absolute alcohol.
2.	Equal parts of toluene and absolute alcohol.	Chloroform until tissue sinks.	Float on the cedar oil.
3.	Toluene.	Fresh chloroform for a short time.	When it sinks replace with fresh oil.
4.	Toluene and paraffin, equal parts (38° C.).	Pure paraffin melted (54° C.).	Pure paraffin melted (54° C.).
5.	Pure paraffin melted (54° C.).	Fresh pure paraffin (melted).	Fresh pure paraffin (melted).

In the use of toluene or xylene, steps 2 and 4 may be omitted, especially with small objects. A second lot of toluene (xylene, benzene) and a change of paraffin are indicated if the object is large. Step 3 may be omitted with small objects when chloroform or cedar oil is used for clearing. The *time* allowed for completion of clearing should be longer rather than shorter, as the effects of insufficient clearing are much more serious than any result of too prolonged a stay in the clearing fluid.

52. Infiltration. As has been indicated above, the clearer is replaced by melted paraffin, which then occupies the interstices of the tissue. Paraffin melting at about 54° C. may be used. It is best not to expose to a higher temperature than is necessary nor for a long period of time, as the heat tends to shrink and toughen, especially if the dehydration (and consequently the clearing) have been incomplete; this is particularly true of organs rich in connective tissue. The paraffin oven should be maintained at a temperature only slightly above that at which the paraffin melts.

If a mixture of toluene (xylene or benzene) and paraffin is used as an intermediate step (step 4) the moderate temperature required (about 38° C.) to keep the mixture fluid may usually be attained by placing the dish on the *top* of the paraffin oven. Large pieces of tissue may be left here as long as 2 days, during which time the toluene slowly evaporates.

After most clearers, it is well to change the pure paraffin once during infiltration. If the block of tissue is large, two changes are indicated. One hour in the pure paraffin usually suffices if the object is quite small. Large objects may require 12 or 24 hours in the pure paraffin.

For hard objects such as entire insects, spiders and other arthropods, it will be necessary to cut a slit in the body wall to permit more thorough infiltration. It may also be advantageous to use paraffin with a melting point of 56–58° C. The use of still harder paraffin made by mixing the paraffin with bay-berry wax is sometimes advisable.

53. Imbedding. It is best to use fresh paraffin for imbedding, and, with a melting point higher than that of the infiltration paraffin, 52–54° C. paraffin answers well in a room of 19–20° C. and will be generally used. If the cutting is to be done in a room of lower temperature, a softer grade of paraffin may be used for imbedding; if at a higher temperature, a harder paraffin should be chosen, as when summer work is necessary.

As a general rule, hard tissues require a harder imbedding paraffin, which is also better when very thin sections are desired. Large sections, which usually must also be relatively thicker, need

a softer paraffin. It is better to work with a paraffin harder than the room temperature itself would call for and then regulate the cutting temperature by placing a source of heat, such as an electric light, nearer or farther away from the microtome knife.

FIG. 2. — Columbia Paraffin Oven

Watch glasses that have been slightly greased with glycerin may be used as molds for paraffin blocks, or paper trays for blocks containing larger pieces of tissue. To make the trays, take a piece of good-quality writing paper of a width equal to the width of the desired tray plus twice its required depth, and of a length equal to the length of the tray plus a little over twice its depth. Fold this paper along the lines *A–A* and *B–B* (Fig. 3). Bring the sides and ends into position and then pinch out the paper along the lines *C–D* so that *AC* will coincide with *BC*. Fold the " dog-ears " thus made back on the ends of the tray. The ends of the tray, now being higher than the sides by a distance equal to *DE*, may be bent down, to prevent the tray from unfolding.

54. In imbedding in paraffin, observe the following rules: (1) Take no more paraffin (no larger box) than is needed to form a mass of convenient size around the specimen. The aim is to have as homogeneous a mass as possible; paraffin tends to crystallize if it cools slowly, hence the smaller the mass the more rapidly it may be cooled. (2) Let the imbedding paraffin when poured into the

box be several degrees above its melting point, and let the tissue have an equal temperature. Should the imbedding paraffin (or the tissue) be too cool it will not set well around the specimen, and a film of air may be enclosed. On the other hand, take care that the paraffin is not hot enough to "cook" the tissue, thereby shrinking it and rendering it hard and tough or ruining it altogether. (3) Cool the paraffin by floating the box on *cold* water. A homogeneous, translucent, paraffin mass can only be secured if

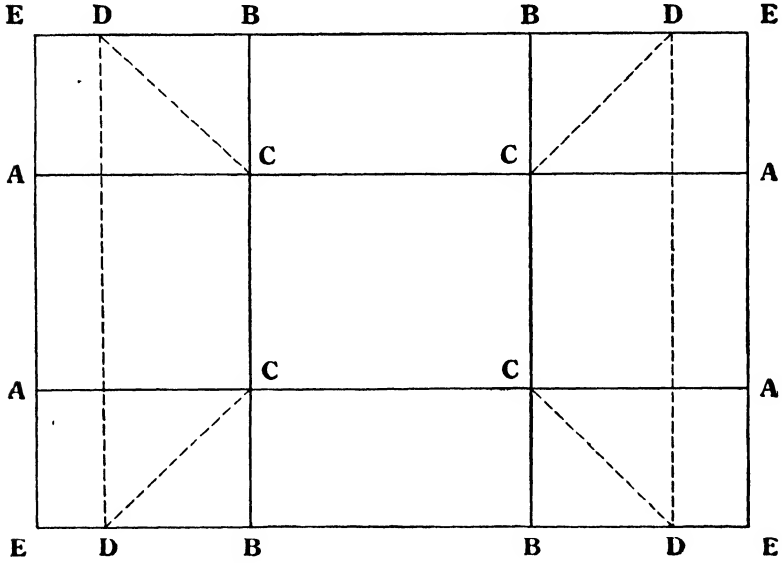


FIG. 3. — Diagram of a Paper Box for Paraffin Imbedding

it is quickly cooled. When a film has formed on the surface strong enough to resist rupture, immerse the block, or drop 95 per cent alcohol upon the surface. Ice is an advantage in summer imbedding if cold water is not available. When neither ice nor cold water is available, good results are sometimes secured by floating the box on a shallow dish of (used) ether-alcohol (§ 63). A homogeneous paraffin is only secured if the paraffin is allowed to shrink in cooling; it is therefore well to make the boxes as shallow as possible, that is, much broader and longer than high. Watch glasses, watch crystals, small tin pans, etc., may be used as imbedding receptacles.

55. Crystallization of the Imbedding Mass. Paraffin that has crystallized is crumbly and will not give good sections. When crystallization has occurred, it is best to reimbed. Its occurrence is usually due to too slow a cooling of the imbedding mass, or cooling under conditions that prevent the paraffin from shrinking. It is sometimes due to the presence of impurities in the paraffin, such as water (?), excess of clearer, etc.

56. Sectioning. The essentials for good paraffin sectioning are (1) well-imbedded tissue, (2) a sharp microtome knife (or section razor), (3) a room of the proper temperature, (4) the paraffin block properly trimmed and arranged in the microtome, and (5) an adequate microtome. Furthermore, tissues fixed and hardened in different ways cut very differently. Tissue fixed in Hermann's, Flemming's, Müller's, Zenker's, or Carnoy's fluid, etc., cuts well; alcohol and mercuric chlorid tissue is more apt to be tough or hard, etc. The different organs, tissues, and animal forms have, of course, very different adaptabilities to the method.

57. The Knife. For cutting sections, one of three types of knives may be used: an ordinary heavy razor, one side of which is plane; a safety razor blade; or the heavy knife which accompanies the standard microtomes. The first-mentioned is less commonly employed now than formerly. When it is used with some types of microtomes, a metal slotted plate may be necessary to support it. The safety razor blade requires a special clamp to hold it in position. Some of the clamps furnished with the microtomes are too blunt on the edge and require grinding down. The blade should be so inserted that only a little more than the bevel edge shows beyond the edge of the clamp. If this is not done the flexible blade may spring and thus result in sections of unequal thickness. For soft tissue this blade, if well stropped, is quite satisfactory. For chitinized material the heavy microtome knife is more suitable. For fine work it should have an edge keen enough to cut a hair held between the fingers at a point $\frac{1}{4}$ inch or more from the finger tips. To obtain such an edge requires careful honing and stropping. Honing is best done upon a yellow Belgian or similar stone followed by a blue-green water hone or a fine Arkansas oil stone. The yellow stone should be lathered with olive-oil soap, the blue-green stone rubbed with water, the Arkansas stone rubbed with thin oil, such as machine oil thinned with xylene. Stones should be kept clean and free from dust.

In honing, the edge should be foremost, the knife held at right

angles to the stone, flat against it, and drawn in such a way that most of the edge comes in contact with the stone in the stroke, rolling the knife over on its back at the end of the stroke. Most blades are so ground as to require a special back while honing. The hand, while holding the blade in sharpening, describes more or less a figure 8 on the fore and back strokes. Use little or no pressure.

The movements in stropping are similar to those in honing, though the knife must, of course, be drawn with the back foremost instead of the edge. Strops fastened to a wooden back are very satisfactory. Gage recommends that the strop be prepared for use by rubbing into the smooth surface some fine carborundum powder (i.e., 60-minute carborundum), diamantine, or jewelers' rouge. Stropping may require hours to obtain the desired result, depending upon the hardness of the knife. A dull blade will cause jamming or wrinkling of the ribbon. One with nicks may tear the tissue or cause a lengthwise splitting of the ribbon. A knife once put in good condition usually does not require prolonged sharpening if it is properly taken care of.[1]

58. The Microtome. A number of standard microtomes are available with which sections from one micron upwards may be cut. In the larger American laboratories some form of rotary microtome is most commonly used. The sliding microtome of the Jung-Thoma type is preferred by some for work of great precision.

59. The Paraffin Block. An object once imbedded in paraffin or celloidin may be left indefinitely without harm. For small objects, the following procedure is best: The block holder is set in an upright position with the flat surface uppermost and upon it some paraffin is placed. The paraffin block, roughly trimmed, is " soldered " in position to the holder by means of a heated dissecting needle. When cold, the block should be closely trimmed, two faces absolutely parallel to insure a straight ribbon. When double imbedding has been employed (§ 72), the paraffin-celloidin block should be trimmed so as not to expose the celloidin on the parallel faces, otherwise ribboning will be prevented. With

[1] In the following papers are described methods and devices for sharpening microtome knives. Apáthy, Zeitschr. f. wiss. Mikr. 14 : 157, 1897; and 29 : 480, 1912; G. M. J., Anat. Rec. 9 : 26, 1915; Malone, Anat. Rec. 24 : 97, 1922; Newton, Journ. Roy. Micr. Soc. p. 166, 1917; Ssobolew, Zeitschr. f. wiss. Mikr. 26 : 65, 1909; Van Walsem, Zeitschr. f. wiss. Mikr. 33 : 341, 1916.

larger objects, the holder may be heated and the paraffin block, properly trimmed with two sides parallel, may be directly melted on.

Clamp the holder in the microtome so that the tissue will be at the proper level for cutting, being careful to have the parallel sides also parallel to the edge of the knife. Use a very sharp, dry section knife for cutting the sections. Clamp it in the microtome

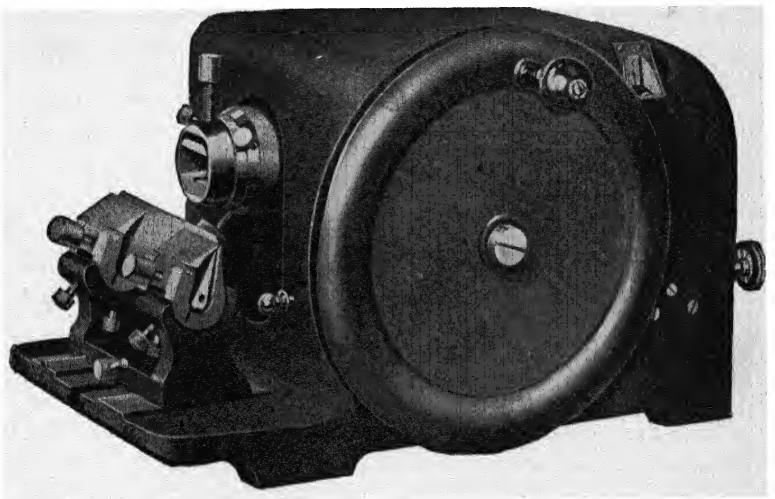


FIG. 4. — Spencer Rotary Microtome

slightly inclined to the cutting surface of the tissue. If the temperature of the room is right for the paraffin used, the sections will remain flat, and if the directions given above for trimming and arranging the block be observed, they will adhere and thus form a ribbon. If the room is too cold or the paraffin too hard, the sections will roll; if it is too warm, the sections will crush or be imperfect. If a microtome in which the knife is not fixed is used, make the sections with a rapid straight cut as in planing. Do not try to section with a drawing cut as used in celloidin sectioning. Ten microns will be found the most convenient thickness for the sections, though in special cases they should be thinner or even thicker. Handle the sections by means of a camel's hair brush, a needle, or sometimes on a scalpel handle or section lifter when cutting ribbons, etc.

60. Electrification of the sections is a frequent source of annoyance in microtomy. It appears to be due mainly to friction in the cutting (see table), the static charge being more troublesome in dry weather, apparently. Obviate the cutting difficulties or await more favorable conditions.

Geiser (Science, 55 : 212, 1922) suggests the following remedy. A thin blade of celluloid (such as one of the 6-inch rulers furnished by the biological supply houses) is screwed against the outer face of the section knife by means of the usual knife-holding screws of the carriage. A long, narrow strip of thin, tough paper is passed up between the celluloid blade and the knife, until about 3 cm. of it protrudes above. After the paraffin block has been properly trimmed and adjusted to the knife, the sections are cut, and as each one is cut it is attracted and held by the paper strip which is pulled along with the fingers so as to produce a series. When the strip is nearly filled with sections, it is taken, and fastened to the table or board with thumbtacks, to keep it from curling, and another strip substituted.

THE CELLOIDIN METHOD

61. A comparison with the paraffin method has already been given (§ 48); there may be emphasized here three points: (1) with paraffin heat is required, with celloidin no heat; (2) paraffin must be removed from the sections subsequently, celloidin need not be and usually is not dissolved out; (3) by the paraffin method may be obtained small sections (5 cm. square or less), and thin, by the celloidin, larger sections, but thicker. With paraffin heat (melting and cooling) is necessary, and the mass is sometimes spoken of as a *fusion* imbedding mass; celloidin is a solution, and the mass is left in the tissues by *evaporation*, or its equivalent.

In the celloidin method the imbedding mass with which the spaces of the tissue are to be filled is collodion, a solution of celloidin or pyroxylin[1] (soluble cotton) in ether and alcohol, hence the

[1] Celloidin and Parlodion are specially prepared and purified forms of pyroxylin or soluble cotton, nitrocellulose. The writers, following Ruby (Anat. Rec. vol. 55, Supplement, p. 74) and Davenport and Swank (Stain Technology, vol. 9, p. 137) have found it distinctly advantageous and inexpensive to employ nitro-cellulose RS ½ sec., made by the Hercules Powder Co. (Wilmington, Del.). Ten per cent and 20 per cent solutions in ether-alcohol (§ 63) may be employed as thin and thick solutions (§ 64).

Trimmings from the celloidin (nitrocellulose) blocks after the alcohol or chloroform hardening may be dried out and redissolved. Since celloidin (nitrocellulose) is markedly inflammable, it is shipped wet with water or moistened with alcohol. If wet, it must be dried before solution in ether-alcohol; if moistened with alcohol, the amount of such alcohol should be taken account of in making the solution.

THE CAUSE OF DEFECTS IN SECTIONING PARAFFIN BLOCKS

Conditions may be due to one or more of the following faults	Section catches on block	Electrification	Object drops out	Variable thickness	Knife scrapes on back stroke	Splitting lengthwise	Rolling	Jams and wrinkling	Scratchy noise	Crooked ribbon
Wedge-shaped section										×
Non-homogeneous paraffin or impurities						×			×	×
Over-heating in oven									×	
Dull knife	×	×					×	×		
Too soft paraffin	×							×		
Too hard paraffin							×			
Tilt of knife too small	×	×		×	×			×		
Tilt of knife too great				×	×	×	×			
Edge of knife dirty	×							×		
Knife nicked		×				×				
Tissue too hard		×		×	×					
Knife blade too thin				×	×					
Imperfect penetration			×							
Clearer not all removed			×							
Weather conditions		×								

steps, which are comparable with those of the paraffin method (§ 48), are (1) *dehydration*, removal of the water; (2) *saturation* with ether-alcohol, the solvent of the celloidin; (3) *infiltration* with celloidin solutions, a thin and a thick; (4) *imbedding* in a thick celloidin mass, which is *hardened* and (5) sections *cut*.

62. Dehydration. Let it be complete, as in the preparation for paraffin imbedding (§ 50). Immerse the tissue in 95 per cent and absolute alcohol for 12–24 hours or longer, changing 1–3 times. Consult also § 50 upon the dehydration of tissue.

63. Saturation with Ether-alcohol (equal parts of pure ether and absolute alcohol). Remove the tissue from the strong alcohol and place it in a stoppered vial of ether-alcohol for 12–24 hours. In addition to preparing the tissue for the collodion solutions, it completes the dehydration, should it be imperfect. In special cases, or if the dehydration is very thorough and the specimen small, this step may be omitted. A satisfactory infiltration is, however, more certain if ether-alcohol be used.

64. Infiltration: (a) with Thin Celloidin. Pour off the ether-alcohol and add the thin (2 per cent) solution of celloidin in ether-alcohol. This, being a solution in ether-alcohol with which the tissue is saturated, readily permeates it. It is best to allow at least a day for this to take place, although if there is time a stay of several days is better, there being little or no danger of deterioration while in the solution. With large (1 cc. +) objects an infiltration of a week or even a month is advisable.

Infiltration: (b) with Thick Celloidin. Pour off the thin collodion solution and add thick (5 or 6 per cent) solution (in ether-alcohol). In this there is gradual concentration of the solution in the tissue. Allow small specimens to remain a day, or, better, several days; larger objects should be given a proportionately longer time, a week to a month, or even longer.

If the object to be imbedded, such as many embryological specimens, is one with large interior cavities with thin walls the transfer from the thin solution to the thick solution may be attended by a collapse of the walls and a consequent shriveling and distortion of the specimen. Avoid this by allowing the thin solution to thicken very gradually by evaporation in a dry atmosphere, as under a bell jar with calcium chlorid present, until the solution has attained the right consistency. To accomplish this it is only necessary to have the cork of the vial containing the specimen perforated by a small hole. A small piece of paper may be inserted with the cork, or with porous corks no special effort need be made. Unless the thick solution has itself thickened by evaporation, with large specimens it is advisable to follow the 6 per cent bath with a stay in a thicker solution, as 10 or 12 per cent, for a day or so.

65. Imbedding. Pour off the 6 per cent or 12 per cent solution and add, for a short time at least, a 12 per cent solution of celloidin (in ether-alcohol). The tissue is now ready for imbedding in 12 per cent, which may be accomplished in either of two ways: (*a*) *on a holder* or (*b*) *in a paper box.* Only those specimens need be imbedded in a box that, from their shape, or for purposes of careful orientation or serial sectioning, require a larger imbedding mass around them.

(*a*) *On a holder* (wooden-block). Choose a block of a convenient size; put a drop or two of celloidin upon one end and insert a pin vertically to the surface near the edge. Transfer the tissue from the vial of thick celloidin to the block and lean it against the pin. The shape of many tissues will obviate the need of a pin. Pour the thick celloidin on to the tissue, drop by drop, moving the block in such a way that the thick viscid mass may be made to surround and envelop the tissue. Continue to add drops of celloidin at intervals until the tissue is well surrounded, and then, as soon as a slight film hardens on the surface, invert the holder bearing the tissue in a shell-vial of large diameter or glass box containing enough chloroform to cover the specimen. Cork or cover so that the chloroform will not evaporate. If the piece of tissue is of awkward size and shape, oiled paper may be wound around the end of the wooden holder and tightly tied, the projecting hollow cylinder being long enough to receive the object. The tissue may be put into the cylinder as before, and the celloidin slowly poured in drop by drop until the specimen is completely covered. When a film has formed, place in chloroform as before.

(*b*) *In a paper box.* When a box is required for imbedding proceed as follows: The inside of the paper box should be slightly oily to prevent the celloidin from sticking to it. Rub upon the paper that is to be folded to form the box a little vaseline, and then with a cloth or lens paper remove as much as possible. Fold the paper into a box of convenient size and shape. Remove the object from the thick celloidin and place it in the box, arranging it in the manner wished with a view to sectioning it later. Pour over it slowly, drop by drop or a little at a time, a 12 per cent solution of celloidin until the specimen is well covered and the box sufficiently filled. It is better to have a deep layer over the specimen. The 12 per cent solution does not afford the best mass for cutting, so that, with large objects, it is better to allow the mass in the box to

thicken by evaporating it slowly under a bell jar (aquarium jar) until it has attained such a consistency that it is no longer fluid.

66. Hardening. When the celloidin mass is so thick that it only dents when touched with the finger nail it is ready for hardening. This may be done by pouring chloroform into the jar in which the imbedded material is placed, covering from the air. The chloroform vapor hardens the mass. When it is well set it may be transferred to a jar of the chloroform, which takes out the ether-alcohol and *hardens* the celloidin mass, for which a few hours is sufficient. Allow the chloroform to act for 6–24 hours. The imbedding mass remains quite transparent when no water is present. The hardening action of the chloroform may be quickened and intensified by carefully heating the chloroform until bubbles of ether begin to come from the specimen. Do not let the chloroform evaporate.

67. Alcohol Hardening. When the celloidin mass is hard, whether clear or not, it may either be transferred to alcohol of about 82 per cent strength in which it is stored until cut, or it may be placed in clarifier (castor oil, 1 part; xylene, 3 or 4 parts). Alcohol of higher percentage softens the mass; lower grades, such as 67 per cent, usually increase the hardness of the celloidin and in some cases are to be recommended.

The choice between alcohol and clarifier involves no decision of importance in technique. The method of clarification has the advantage that the orientation of the specimen in the microtome preparatory to cutting can be more perfectly done. If the tissue has been stained *in toto* (§§ 79, 144) the sections may be mounted directly from the clarifier as soon as cut. Any mercuric chlorid precipitate (§ 11) that may be present can be dissolved out by means of a solution of iodin in the castor-xylene. The castor oil, however, renders the microtome, knife, hands of the operator, etc., sticky, and the method is not so cleanly as the alcohol method. On the other hand, alcohol tends to rust microtome and knife. Clarification is preferable for serial work in celloidin.

68. Clarification. Celloidin blocks, transferred from the chloroform hardener to an oil mixture such as castor-xylene (§ 67), will become quite transparent (clarified) and hardly discernible, so that the tissue is readily seen. Sometimes, however, the celloidin remains white and opaque, due to the presence of moisture, and considerable time is required for its clarification. In such cases the process may be hastened by placing the tissue in the clarifier in a warm place, and changing the clarifier several times. If the block still remains opaque, remove to 95 per cent alcohol for a day

for dehydration, pass through chloroform, and into clarifier. In this way the mass may usually be cleared perfectly. Change the clarifier to fresh after the first day or so. The sectioning may be done after a few hours' immersion, although a several days' clarification is preferable.[1]

Cutting Dry. Cedar oil (thin) may be employed for clearing celloidin blocks instead of the castor-xylene. Use first a mixture of chloroform and the oil, replacing it with pure cedar oil. Since cedar oil is not a solvent of celloidin as is castor oil, celloidin blocks cleared in this way may be cut dry or stored in stoppered bottles for subsequent cutting. Such blocks, however, tend to darken with age.

69. Cutting the Sections. There is no marked difference between the sectioning of celloidin blocks preserved in alcohol and those that have been clarified. In the following paragraphs 67 per cent alcohol should in the reading be substituted for clarifier if alcohol was used in the hardening.

If a paper box was used, after the celloidin is ready for cutting, remove the paper, trim the block as is desired (see below), dry the base of the block with a cloth, put some 6 per cent celloidin upon the wooden block or other holder, and press the base of the celloidin block firmly against it; within a few minutes it will be firmly cemented and one may proceed at once to clamp the holder in the microtome and commence cutting.

For celloidin sectioning, a long drawing cut is necessary in order to obtain thin, perfect sections. The knife should, therefore, be set at an obliquity of 15–20° or less, so that half or more of the blade is used in cutting the section. Recall that in the paraffin method the knife is usually to be set at right angles to the direction of the cut, and the stroke is a rapid straight one. Trim away the surrounding celloidin mass, leaving enough, however, to serve as a support to the tissue and prevent its bending under the impact of the knife; if the celloidin mass is too tapering, bending will occur and thin sections cannot be cut. To avoid this the celloidin block

[1] The imbedded object may remain in the castor-xylene clarifier indefinitely without harm. The celloidin grows somewhat tougher by a prolonged stay in it. After cutting all the sections desired at one time, the imbedded tissue is returned to the clarifier for future sectioning. It should be remembered, however, that pure castor oil is a solvent of celloidin; hence it is necessary to have the container tightly stoppered, otherwise the volatile xylene will evaporate, leaving the castor oil behind.

is best trimmed in the form of a four-sided truncated pyramid with as broad a base as possible.

Clamp in the jaws of the microtome, placing it so that the mass of celloidin is opposite the side to which the pressure of the knife is applied in cutting. It is advantageous also to have the object placed with its long diameter parallel with the edge of the knife.

When knife and tissue are properly arranged, wet the tissue well with clarifier or alcohol, as the case may be, and flood the knife with the same. Make the sections with a slow, steady, motion of the knife. With a small object (3 × 5 mm.) and a good sharp knife, sections 6–10 microns can be cut without difficulty. In addition to a sharp knife, however, there are necessary well-infiltrated tissue and a hard, firm mass. If serial sections are not desired, it may be more expeditious to cut dry and with a rapid stroke.

70. Transferring the Sections. If the sections are quite thick they may be transferred from the knife to a slide or a dish by means of forceps or a brush; if they are thin, however, it is better to handle them by means of an absorbent tissue paper, as follows: Flood the sections well with clarifier and then by means of a pipette remove the clarifier from the knife and place over the sections the end of a piece of the tissue paper, pressing it down upon the sections if necessary. Carefully pull the paper off the edge of the knife; the sections will adhere to the paper. Place the paper, sections down, on a slide, taking care that the sections are in the desired position. With the finger, carefully press the sections (through the transfer paper) to the slide, and then lift the paper, with a rolling motion, from the slide; the sections will adhere to the slide. Should they stick to the paper instead, lower the paper again, and again firmly press the section to the slide. For further procedure see §§ 137, 138. If it is not desired to mount the sections upon a slide immediately, or if they are to be kept in bulk, as for class work, the transfer paper may be shaken gently in a dish of clarifier or 95 per cent alcohol and the section (or sections) will float free and sink to the bottom.

71. Serial Sectioning. If it is desired to mount the sections in series, proceed as follows: With a camel's hair brush or needle draw the first section, when cut, up toward the back of the knife and make the next section. Place this section to the right of the first, and so on, arranging them in serial order, section after section,

and line below line, until enough are cut to fill the area that the cover glass will cover. Flood the sections as before, by letting the clarifier flow over them, being careful, however, not to float them from their places. Absorb the clarifier from the knife with a pipette, and place over the sections a piece of the transfer paper twice the width of a slide; press it down if necessary, and slowly draw it off the edge of the knife. Should it then be seen that some of the sections are adhering to the knife instead of the paper, it means that the clarifier had been allowed to thicken[1] on them, cementing them to the knife, and the preliminary flooding, to insure their being free, was insufficient. In that case it is best to flood the paper with clarifier, carefully lift it, arrange the sections again, flood them with clarifier, place a clean piece of transfer paper over them, and try again. One soon becomes accustomed to the behavior of the sections, and accidents are rare. In cutting a series of many small sections, some time is consumed and it is necessary to flood the sections on the knife frequently with clarifier while cutting in order to prevent the clarifier from thickening and cementing them to the knife.

THE PARAFFIN-CELLOIDIN METHOD

72. This method is particularly useful in cutting material which is not homogeneous but in which some constituents are harder and more brittle than others, as in the case of the body wall of insects with its underlying parts and in the case of yolk-rich eggs. The material is infiltrated with thin celloidin as in the preceding method, then with medium celloidin, and finally with 8 per cent celloidin, being allowed to remain in each at least 24 hours. Next pour the celloidin containing the object into a watch glass, orienting with a needle. Set the object under a bell jar in chloroform vapor for an hour or two, or until the celloidin is sufficiently hard to permit trimming into small cubes but little larger than the object contained therein. Place the celloidin cubes in a watch glass with chloroform to which 40° C. paraffin

[1] If one is a long time cutting a series of sections, it sometimes occurs that the xylene evaporates, leaving the castor oil, which is thick and viscid and also a solvent of the collodion, so that the sections are not easily transferable but stick rather firmly to the knife. In such a case, fresh clarifier or even a little xylene to dissolve the castor oil must be used.

parings, about equaling the chloroform in bulk, have been added. Leave for several hours, or perhaps over night, at 40° C. Replace the paraffin with 56° C. paraffin, changing once or twice during the course of an hour or so. Place the objects in molds or watch glasses which have been slightly greased with glycerin, and cover with fresh paraffin. Allow the objects to remain a few minutes longer in the oven, then remove and cool quickly. The celloidin cubes show through the paraffin sufficiently to permit orientation. The paraffin blocks should not be trimmed so closely as to expose the celloidin, otherwise ribboning will be prevented. From this point on, the objects may be treated like paraffin-imbedded material.

Small objects may be of such shape that they must be cleared in order that they may be properly oriented. In this case the following method, a modification of one described by Entz (Arch. Protistenkunde 15 : 98, 1909) has been found useful. Make up clove-oil celloidin solutions as follows: Take 8 to 10 grams of celloidin (or parlodion), wipe dry, rinse in 95 per cent alcohol, then soak in absolute alcohol for some minutes to insure thorough dehydration. Remove the celloidin from the alcohol into 100 cc. of a mixture of equal parts of ether and absolute alcohol until dissolved. Now make up two clove-oil celloidin solutions, one with two parts of clove-oil to one of the celloidin mixture, the other with one part of clove-oil to two parts of the celloidin. These should be allowed to stand tightly corked for a day or two to insure homogeneity. Dehydrate the object which is to be imbedded, clear in clove-oil, then transfer first to the weaker celloidin mixture, then to the stronger for several hours each. A longer stay in the celloidin will do no harm. Take a piece of glass, say 18 by 25 mm., dip it into melted paraffin to coat it. When cold, place upon the glass a drop of the thicker celloidin mixture. The cleared object may now be oriented in this drop, its longitudinal axis coinciding with the long dimension of the glass. Place the glass under a bell jar, placing a dish containing chloroform under the bell jar also. In a short time the drop will have hardened sufficiently to permit its being trimmed. The longitudinal axis of the trimmed celloidin should be made to coincide with the long dimension of the glass. After trimming in the other dimension, put the trimmed block in a watch glass for a half hour or more with some chloroform; add paraffin shavings, and leave for an hour or more in the paraffin oven. Next transfer the celloidin block to a glycerine-greased watch glass filled with fresh paraffin, and, after a few minutes in the oven, remove to a dish of cold water to cool. The subsequent steps are the same as for paraffin imbedding. Methyl benzoate may be used in place of clove-oil for clearing and in the celloidin mixture.

A number of other paraffin-celloidin methods have been described, some of them especially adapted to small objects which

are difficult to orient. The following papers may be consulted. Apáthy, Zeitschr. f. wiss. Mikr. 29 : 449, 1912; Church, Science 47 : 640, 1918; Dahlgren, Journ. Applied Micr. 1 : 67, 1898; Hoffmann, Zoöl. Anz. 56 : 142, 1923; Jordan, Zoöl. Anz. 16 : 33, 1899; Kornhouser, Science 44 : 57, 1916; Mayer, Zeitschr. f. wiss. Mikr. 33 : 3, 1916; Newth, Quart. Journ. Micr. Sc. 63 : 545, 1919; Péterfi, Zeitschr. f. wiss. Mikr. 38 : 342, 1921.

THE FREEZING METHOD

73. This method is expeditious and of use in the rapid examination of tissues, and therefore especially serviceable in the pathological laboratory and in clinical diagnoses. It may also be used in cutting tissues that are too hard to be cut satisfactorily by means of either the collodion or the paraffin methods, and in the examination of tissues for substances (e.g., fats) which the solutions necessary for the paraffin and celloidin methods dissolve out. Both fresh and fixed tissue may be cut by means of the freezing microtome and with or without any special mass such as is used in paraffin or celloidin imbedding. Some histologists quite prefer the freezing method to the paraffin or celloidin methods for general use.

When no mass is employed the tissue is simply frozen and cut, or, if it is fixed tissue, soaked well in water first and then frozen. When extreme haste is not so essential it is better first to saturate the tissue with some solution that does not crystallize on freezing, but simply hardens, since the formation of the ice crystals is hurtful to the tissue. Such are solutions of *gum arabic* or *sugar* and *anise-seed* oil, and they are spoken of as *congelation masses.*

74. Infiltration. Gum arabic or anise-seed oil may be used. (*a*) *Gum arabic.* If the tissue has been fixed and is in alcohol, remove the alcohol by soaking it for several hours to 1 day in water. Remove to a thick solution of gum arabic in water, in which it may remain for about 24 hours. It is then ready to freeze and cut.

(*b*) *Anise-seed oil.* For this method the tissue should be first dehydrated (§ 50).[1] When dehydration is complete, transfer the tissue to anise-seed oil, in which it may soak for 12–24 hours; it is then ready to freeze and cut. It is particularly adapted for use with tissue that has been stained *in toto.*

[1] Anise-seed oil will, however, clear from 90 per cent or even 82 per cent alcohol; this is sometimes of advantage.

75. Cutting. Place a drop of the solution of gum arabic (or anise-seed oil) upon the object carrier of the freezing microtome and turn on the carbonic acid (or ether) spray. When the mixture begins to harden, place the object upon it in an abundance of the solution and freeze it nearly solid. Covering with an inverted cup hastens the freezing. An especially wedge-shaped knife is necessary because of the hardness of the mass.

When the tissue is completely frozen, cut it with a straight movement of the knife, as in the paraffin method, holding it firmly upon the knife rest and making the strokes as rapidly as possible, at the same time rapidly raising the tissue a few microns at a time by means of the microtome screw. There are a number of automatic microtomes specially designed for use with the freezing method.

The mass of sections is transferred to a dish of water in which the gum arabic is dissolved away, and the sections are ready for staining (§§ 139, 148). If anise-seed oil is used, the sections are to be transferred to 95 per cent alcohol which will dissolve out the oil; if the tissue has been stained *in toto* the sections may be transferred to anise-seed oil (or other clearer) and mounted in balsam directly.

76. Rapid Method. Blocks of tissue 1 cm. thick should fix in 10 per cent formalin 12–24 hours. If haste is a factor, take thinner pieces and fix for 1 minute or more. Trim the block so that it is about 5 mm. thick; rinse in water for a few seconds, transfer to the freezing microtome, freeze, and section.

Float the sections, when cut, from the knife into water, from which they may be gotten upon the slides by means of a camel's hair brush. Drain off the water and press the sections out smooth by means of blotting paper, filter paper, or other absorbent paper. Cautiously drop over the sections 95 per cent and absolute alcohol and follow this immediately with thin ($\frac{3}{4}$ or $\frac{1}{4}$ per cent) celloidin solution (§ 64) which, when it has partially evaporated out, will serve to support the section and fasten it to the slide. It is now ready for staining. (§ 146.)

76a. Dry Freezing. For the application of the Altmann dry freezing method in the study of cell structure, consult I. Gersh, Anat. Rec., vol. 53, p. 309, 1932.

here to the albumen fixative when well pressed down, it can in many cases be made to do so by briskly rubbing the reverse side of the slide with a woolen or silk cloth.

142. One-half Per Cent Celloidin (collodion). The adhesion of sections that are particularly valuable or relatively thick may be insured by treating them with ½ per cent celloidin, as follows: Fasten the sections to the slide by either of the above methods, remove the paraffin by xylene (§ 145), and then, after draining off the xylene from the slide, for 10–13 seconds, put it into a bottle containing ½ per cent celloidin. In a minute or more the celloidin displaces the xylene and penetrates the sections. The slide is removed, allowed to drain for half a minute and then put into a jar of 67 per cent alcohol which sets the celloidin [20]. Care must be taken that the sections do not dry. It is now ready for the staining processes (§ 146–).

PRELIMINARY STEPS

143. These are somewhat different for paraffin and celloidin sections. In the case of the former, it is necessary to remove the paraffin by means of a solvent (e.g., xylene), remove the paraffin solvent by alcohol, and usually remove the alcohol with water. In the case of celloidin sections, the first step is unnecessary. If the tissue was hardened and cut in alcohol, the second step may likewise be omitted.

144. *In Toto* Staining. If the staining has already been done (§ 79), these " preliminary steps " are, of course, all unnecessary, save the removal of the paraffin by xylene.

145. Xylene. Leave paraffin sections in xylene until the paraffin is entirely dissolved out. This usually requires only a few seconds. A longer stay generally does no harm.

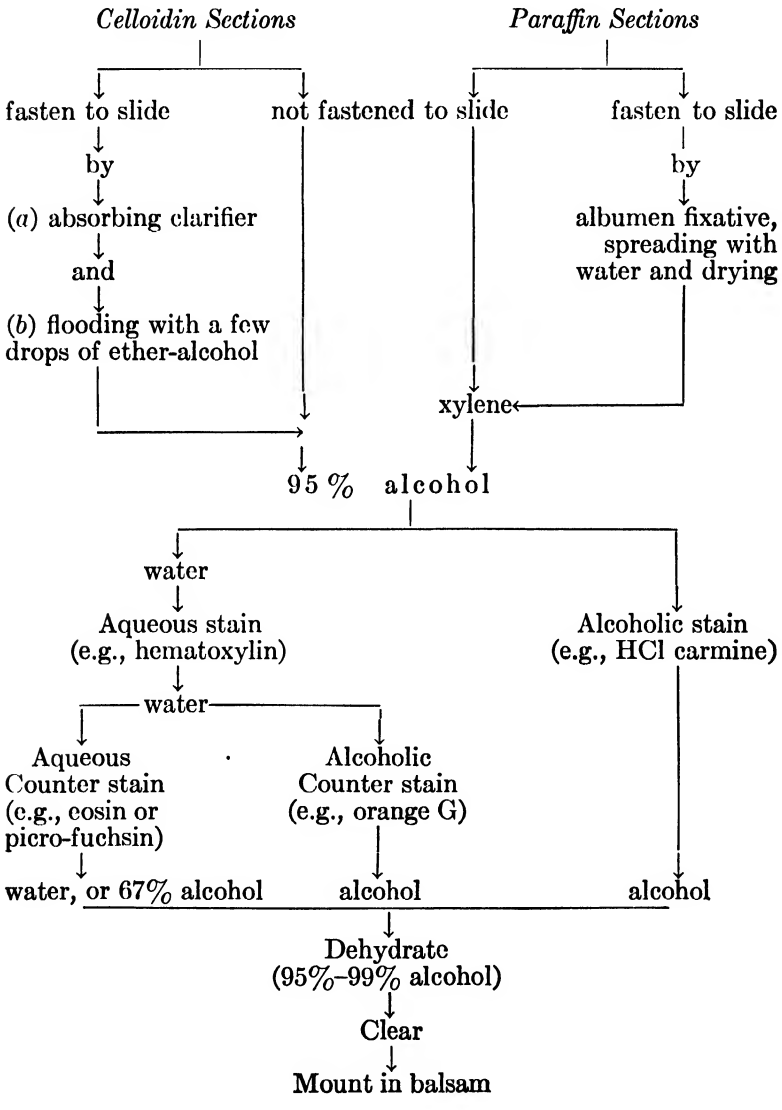
146. Alcohol. Transfer paraffin sections from xylene to 95 per cent alcohol, leaving the sections in the alcohol for 5–10 minutes; or, if you wish, shorten the period to a minute or so by waving the slide gently to and fro in the alcohol.

Celloidin sections cut by the clarification method are placed in alcohol to remove the clarifier. This may take a longer time, and if there are many slides it is well to use two changes of alcohol. A longer stay in alcohol does no harm.

147. Water. Remove the 95 per cent alcohol with water if the stain is an aqueous one.

148. Staining. The following schema shows the general steps in staining and mounting. In all the processes, seemingly com-

SCHEMA FOR THE STAINING OF SECTIONS



plicated, if it is remembered that the succession of media in histological technique generally depends upon their miscibility or some special reaction, and if the *reason* for the various steps is recognized, much of the difficulty in remembering the order in which they come will be avoided.

149. Whether stained or unstained, prepared for microscopical examination by isolation or sectioning, and especially if it is desired to keep the preparation, it is necessary to *mount* it in some way, i.e., so arrange it upon some suitable support (glass slide) and in some suitable mounting medium that it may be satisfactorily studied with the microscope.

Mounting may be:

I. Temporary, or
II. Permanent, — as
A. Dry, or in air,
B. In a medium miscible with water, or
C. In a resinous medium, in which case it is necessary first to remove all water by either (*a*) drying — *desiccation*, or (*b*) a series of displacements, i.e., (1) Removing the water with strong alcohol — *dehydration;* (2) Removing the alcohol with clearer — *clearing;* (3) Replacing the clearer with balsam or other resinous mounting medium.

150. Temporary Mounting. Illustrations may be found in the examination of blood corpuscles and living ciliated cells (§ 39). Temporary examination of tissues is quite simple, though important, and for this it is only necessary to place the teased tissue or section on the slide in a drop of the fluid in which it is at the time, normal salt solution, dissociator, or alcohol, and cover. The examination of preparations intended for permanent mounts during the staining or before mounting will often serve to detect faulty treatment at a time when it may be remedied without great expenditure of time, or to discard the specimen as worthless.

151. Indifferent Media. These are of much wider usefulness, and are numerous. There may be mentioned:

(1) *Physiological salt solution.* Distilled water, 100 cc.; common salt, 0.7 gram. As applied to vertebrates, it is customary to take, for amphibia, 0.6 gram; reptiles and birds, 0.75 gram; mammals, 0.9 gram, of the sodium chlorid.

(2) *Ringer's solution* (modified). Sodium chlorid, 0.80 gram; calcium chlorid, 0.02 gram; potassium chlorid, 0.02 gram; sodium bicarbonate, 0.02 gram; distilled water, 100 cc.

152. Permanent Mounting. This usually includes (*a*) mounting dry on a ring or in a cell, (*b*) mounting in glycerin or glycerin jelly, media miscible with water, and (*c*) mounting in Canada balsam or damar, resinous media.

153. Mounting Dry. The preparation may be either upon the under side of the cover glass (best, if possible) or it may rest upon the bottom of the cell.

In the first case a shallow cell made by a shellac ring will be sufficient; in the second, a shellac ring may not give a deep enough cell, and a paper, hard-rubber, or metal ring may be cemented to the slide.

154. Rings on Slides. When the object is thick, the cover glass may be supported by three bits of glass of a thickness about equal to that of the object. A neater method is to spin a ring of shellac cement on the slide placed on the turntable. By repeatedly ringing, a cell of considerable depth may be made. Shallow cells may be made of balsam. A balsam cell will remain hard long enough for the balsam under the edge of the cover to stiffen sufficiently to support the cover. Rings of hard rubber or of glass of different thicknesses may be purchased from dealers. These are fastened to the slide with balsam or cement.

(*a*) *When the preparation is on the cover.* Prepare a shellac cell (§ 156) on the slide, of a size slightly smaller than the cover to be used, and allow it to dry for a day or so. Warm the cover bearing the preparation, to remove the last traces of moisture, and place it, film side down, upon the ring. Warm the slide until the edge of the cover may be made to adhere to the shellac ring, and press the cover down until it adheres all the way round. Seal the cover with shellac, and label.

(*b*) *Mounting in a paper or rubber cell.* With a brush, cover one side of the ring with a layer of shellac and place it on the center of the slide, shellac side down; place within the cell the preparation, arranging it in the manner desired, and place upon the ring a cover glass of a suitable size, and seal it with shellac; label.

155. Mounting in Glycerin Media. (*a*) Pure glycerin; (*b*) glycerin and acetic acid, 1 per cent; (*c*) glycerin and a stain. As glycerin extracts most stains (except carmine) it is sometimes advisable to have a small amount of stain dissolved in the glycerin used for mounting. Of such combined mounting and staining

mixtures may be mentioned (1) glycerin and Congo red, (2) glycerin and carmine. Other combinations may be used.

156. Congo Glycerin. *Formula:* ½ per cent aqueous solution, Congo red, 1 part; glycerin, 1 part.

157. Carmine Glycerin. *Formula:* Carmalum, 25 cc.; glycerin, 75 cc.

158. Mounting in Glycerin Jelly. The preparation should be mounted from some aqueous solution. Warm the slide gently and put it upon the centering card; in the center of the slide place a drop of warmed (melted) glycerin jelly. Remove the object from the water or aqueous solution and arrange it in the glycerin jelly. Grasp a cover glass with the fine forceps, breathe on the lower side, gradually lower it upon the object and gently press it down. Allow the glycerin jelly to set, keeping the slide horizontal meanwhile. Scrape away the superfluous glycerin jelly around the cover glass and seal with shellac (§ 165).

In mounting in glycerin media it should be appreciated that included air bubbles are permanent; hence pains should be taken to exclude them.

159. Mounting in Balsam. *By desiccation.* Certain preparations may be mounted in balsam, by drying, the method of desiccation, e.g., cover-glass preparations of bacteria, stained cover-glass preparations of blood, etc. For this it is only necessary that the preparation be absolutely dry, and that a small drop of balsam be placed upon it or upon the under side of the cover glass, which is carefully placed over the specimen and pressed down.

Mounting in Balsam. *By displacement.* Mounting in balsam by desiccation is serviceable for but few preparations in histology, and in most cases the removal of the water by a series of displacements is resorted to. For this the following steps are necessary: dehydration, clearing, mounting in balsam.

160. Dehydration. The sections are entirely freed from water by the use of 95 per cent or absolute alcohol. The slide or free section may either be placed in a jar of alcohol or alcohol from a pipette or may be poured over it. Treat the preparation to be mounted for 5–15 minutes. The thicker the section the longer the time required; celloidin sections require a longer time than paraffin sections. In any case, be sure that the dehydration is complete, giving a longer rather than a shorter time, and then clear.

161. Clearing. This is accomplished by putting the slide in a jar of clearer or dropping the clearer upon the section from a pipette. When the section is cleared it will be transparent. Test it by holding it against a dark background; if it is not cleared it will be cloudy, white, and opaque. Carbol-xylene (melted carbolic acid, 1 part; xylene, 3 parts); xylene; or certain essential oils (origanum, thyme, cajuput, bergamot) are used. See also § 51.

162. Mounting in Balsam. Drain off the clearer and allow the section to stand until there appears the first sign of dullness from evaporation of the clearer from the surface. Then place a small drop of balsam upon the section or upon the cover glass which is then inverted over the specimen.

Remember that in mounting in this way you must always "*dehydrate, clear, and mount in balsam,*" and that the three steps are inseparable.

Natural balsam is acid in reaction, due to organic acids contained. As these bleach basic dyes, notably hematoxylin and methylene blue, it is well to use for most purposes balsam that has been dried out and redissolved in a known solvent, such as xylene or toluene. Neutral balsam solutions are to be preferred. Alkaline balsam is sometimes preferable for some hematoxylin stains; acid balsam in certain other cases (fuchsin acid, injection masses, §§ 119, 232–). Furthermore, such solvents as xylene readily oxidize with the formation of acid products. For delicate work, therefore, it is probably advisable to use as thick balsam as is convenient, and avoid inclusion of air bubbles in the mounting. Benzene balsam from this standpoint is preferable to xylene balsam. Thick solutions should be employed, as it dries out with marked rapidity. For other solvents of balsam, see page 19.

Damar balsam. This medium may be used in the same manner as Canada balsam. Its index of refraction is slightly lower than that of Canada balsam. Damar mounts, while they do not darken with age as do balsam mounts, sometimes become cloudy.

Euparal. A preparation of paraldehyde, eucalyptus, camsal, and sandarak. It has a lower refractive index than Canada balsam, which is an advantage for mounts of whole insects. Its chief value lies in the fact that objects may be transferred directly from 95 per cent alcohol or even 70 or 80 per cent alcohol without the intermediary of a clearer. Whether preparations mounted in Euparal keep the color as well as those mounted in balsam is still an open question. Some of the authors' preparations made several years ago show a tendency to fade. Dobell (Arch. Protistenkunde 34 : 145; 1914) has made a similar observation. For a substitute for Euparal see Shepard (Trans. Amer. Micr. Soc. 37 : 131, 1918).

163. Sealing Balsam Mounts. After the balsam has hardened around the margin of a round cover glass, the superfluous balsam

may be scraped off with a knife and the slide carefully cleaned with a soft cloth moistened with xylene. To give the slide a neater appearance, it may be sealed with either Brunswick black or gold size. To do this, center the slide on a turntable, rotate the table, and with a vertically held camel's hair brush, lightly charged with sizing, strike a ring around the cover. When dry, repeat the operation. Balsam mounts, especially when thick, yellow with age unless sealed.

164. Sealing Glycerin-mounted Specimens. Wipe away the superfluous glycerin as carefully as possible with a moist cloth or a piece of lens paper. Place four minute drops of cement carefully at the edge of the cover at the four quarters and allow them to harden for half an hour or more; these will anchor the cover glass, and the preparation may then be placed upon the turntable and a ring of shellac cement put round the edge while the turntable is being revolved.

165. Sealing Glycerin-jelly Mounts. Allow the glycerin jelly to harden for 12 hours or longer. With a knife scrape away the superfluous jelly and then carefully wipe around the cover glass with a cloth moistened with water. Place the slide on a turntable, carefully center the cover glass, and with a brush seal the edge of the cover by a ring of shellac while revolving the turntable. A second coating may be given subsequently if needed, after the first has dried.

LABELING MICROSCOPIC SLIDES

166. Every permanent microscopic preparation should be carefully and neatly labeled in ink. The label should furnish at least the following information:

EXAMPLE

(1) The number of the preparation, the thickness of the cover glass and of the section.	No. C. 15. S. 10μ.
(2) The name, kind, and source of the preparation.	Ileum of Cat. Transection.
(3) The fixer and the stain.	Z.[1] H. & E.
(4) The date of the specimen.	November, 1898.

[1] It is convenient to adopt a standard system of abbreviations, thus: Z. = Zenker's fluid; He. = Helly's fluid; M. = mercuric chlorid, etc.; H. = Hematoxylin; E. = eosin, etc.

In the case of specimens with which it is advantageous to have more information at hand, a second label may be placed upon the other end of the slide, and it may bear the following information:

(1) Mode of fixation (detail).
(2) Imbedding method.
(3) Stains employed (detail).
(4) Mounting medium (generally not necessary).
(5) Special purpose of the preparation.

A catalogue giving the full data of the specimen, — age, condition of the animal, mode of preparation in detail, special points illustrated, etc., is valuable, particularly in special investigations and with standard specimens.

In the case of whole mounts, there should be written upon the label the name of the object, the locality, date of collection, and name of the collector. For sections the data should consist of the serial number, name of the species, the organ or part, the thickness of the section and of the cover glass, the stain, the fixing fluid, etc. The serial number should correspond with that of the note book or card on which is recorded more complete data concerning the specimen and the technique employed. It is a good practice to label permanent preparations, by means of a writing diamond, with the record number at least.

167. Slide Labels. Gummed labels, ruled or unruled, with or without printing, may be purchased from dealers. If they have a tendency to come off after drying, the fault is due in most cases to greasy slides rather than to defective glue. Some workers prefer to coat one end of a slide with a layer of thin Canada balsam or with varnish thinned with xylene. After drying, this may be written upon with India ink. Later, another coat of balsam or shellac may be applied, and, if desired, covered with a square cover glass. It is a matter of choice whether the label be placed on the left or right end of the slide.

SLIDES AND COVERS

The slides and cover glasses used in histological work are often slightly greasy and should be cleaned before using. After they are cleaned they should be handled by the edges only, or with forceps.

168. Cleaning Slides. For ordinary work it is enough to wipe the slides out of clean water to which about 5 per cent ammonia has

been added. A clean glass towel, free from lint, should be used, and after the slides are cleaned they should be stored in covered glass jars away from the dust. Slides may also be effectively cleaned with soap solution, or they may be placed in 5 per cent solution of Bon Ami in distilled water, from which they may be taken, the Bon Ami allowed to dry on them, stored, and wiped clean when needed. (Gage, 20.)

169. Cleaning Cover Glasses. Place them in 95 per cent alcohol to which 1 per cent of hydrochloric acid has been added. A clean, soft cloth, such as an old linen handkerchief, gauze, etc., should be used for wiping them. In wiping a cover glass "grasp it by the edge with the left thumb and index. Cover the right thumb and index with the cleaning cloth; grasp the cover between the thumb and index and rub the surfaces, keeping the thumb and index well opposed on directly opposite faces of the cover so that no strain will come upon it, otherwise the cover is liable to be broken." (Gage, 20.)

Cover glasses, when cleaned, should be stored in covered glass boxes, or in Petri dishes.

170. Cleaning Mixture for Glass. For special purposes, such as when the slide or cover glass is to be used in the preparation of blood smears, the cleaning mixture whose formula is given below may be used. Place the cover glasses in this mixture, one by one, and permit them to remain over night or longer. Rinse them thoroughly in running water until all color of the dichromate has disappeared, rinse them again in distilled water, and transfer to 95 per cent alcohol, out of which they may be wiped.

Dichromate Cleaning Mixture. Formula: Potassium dichromate, 200 grams; water, 800 cc.; strong sulphuric acid, 1200 cc. Dissolve the dichromate in the water by the aid of heat, and to the solution add slowly the sulphuric acid. The two fluids should be mixed in a lead-lined kettle [20].

171. Used Slides and Cover Glasses, vials, and other glassware that have been used with balsam, cedarwood oil, or other oily substance, may be cleaned by boiling with a solution of strong soap, such as "gold dust," one or more changes. Used xylene or toluene is sometimes useful. Slides and covers may require a second cleaning with the cleaning mixture. If only water, glycerin or glycerin jelly has been used on them, they may be cleaned with water, preferably warm water, and then, if necessary, wiped out of 50 per cent alcohol.

172. Measuring the Thickness of the Cover Glasses. With the cover-glass measurer, determine the thickness of the cover glasses and sort them into three groups: (*a*) those with a thickness of .13–.17 mm., (*b*) those less than .13 mm., and (*c*) those thicker than .17 mm. Groups (*a*) and (*b*) only should be used; (*c*) should be discarded or used only with objects for low magnification.

It is advantageous to know the thickness of the cover glass on an object for the following reasons: (*a*) That one may not try, in studying the preparation, to use objectives of a shorter working distance than the thickness of the cover glass [20]; (*b*) in using adjustable objectives with the collar graduated for different thicknesses of cover, that the collar may be set at a favorable point without loss of time; (*c*) for unadjustable objectives, that one may select the thickness of cover corresponding to that for which the objective was corrected [20]. Furthermore, if there is a variation from the standard, that one may remedy it in part at least by lengthening the tube if the cover is thinner and shortening it if the cover is thicker than the standard [20].

THE MICROSCOPE AND ACCESSORIES

Only the briefest account can be given here of the microscope and its manipulation. For more complete directions the reader is referred to the works of Gage (1920), Carpenter-Dallinger (1901), Guyer (1924), Bausch (1906), and others.

Among the microscopes most commonly found in American laboratories may be mentioned the Bausch and Lomb, the Leitz, the Spencer, and the Zeiss. The draw tube should be pulled out by a gentle spiral motion to the standard length for which the objects are corrected, due allowance to be made for the depth of the nosepiece. The standard length is 160 mm. for the makes noted above, and is indicated by a mark on the tube. For the lower powers of objectives it is not very significant, but for

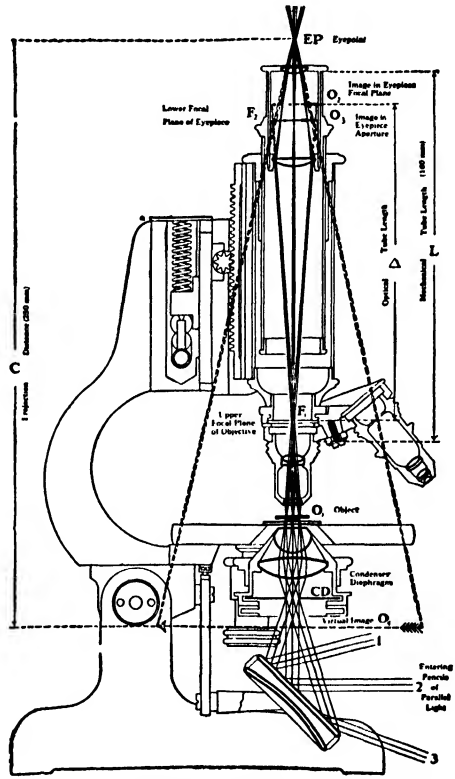


FIG. 6. — Bausch and Lomb Microscope

the higher powers a proper tube length is highly important for clear definition.

173. Focusing. The slide on which the object is mounted is placed on the stage so that the object comes as nearly as possible over the center of the opening. The lowest-power objective is now brought into position, and the tube lowered until the front of the objective nearly touches the glass. This can best be observed by lowering the head to the level of the stage and looking between object and cover glass toward a window. Now look through the eye-piece and slowly focus upwards with the coarse adjustment until the image appears. Center the object carefully by moving the slide. Turn the next higher objective into position. If lenses of different makes are used, it may be necessary to raise the tube a little by means of the coarse adjustment to prevent the objective striking the glass. Objectives that make up the regular outfits are so adjusted as to be parfocal, in which case the tube need not be raised. Next lower the tube until the objective nearly touches the glass as directed for the lower power. Look through the eye-piece and slowly focus upwards with the coarse adjustment until the image appears, then use the fine adjustment. Again center the object if necessary. Repeat the process with the higher powers. Never *lower* the tube while looking through it. The

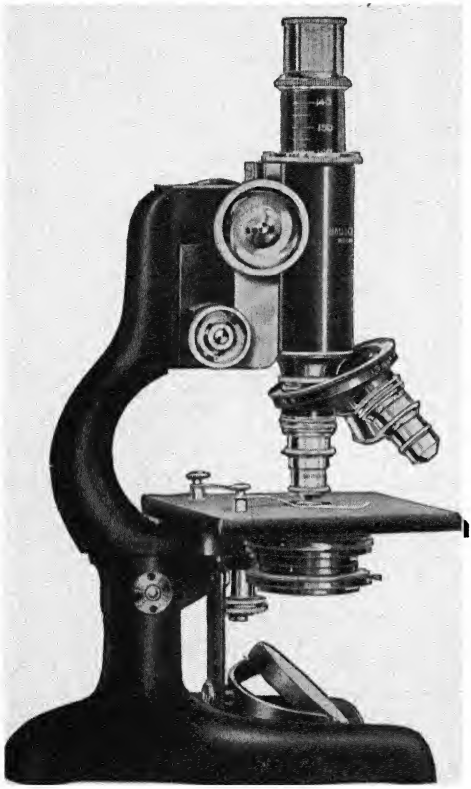


FIG. 7. — Bausch and Lomb Microscope FFS

reason for the procedure of first using a lower power as a finder is obvious when one reflects that the entire field, in using a X-10 ocular with a 3-mm. objective, is only 0.22 mm. in diameter, while with a 32-mm. objective it is nearly 4 mm.

FIG. 8. — Spencer Combination Binocular and Monocular Microscope

Oil-immersion objectives require immersion cedar oil between lens and cover glass. Care should be used to keep lens and oil free from dust. The oil may be wiped off with lens paper. Should

the oil be allowed to dry through carelessness, it may be removed by using lens paper moistened (not wet) with xylene or chloroform.

The higher-grade microscopes are furnished with substage condensers. The usual Abbé condenser, corrected neither for chromatic nor spherical aberration, serves very well for all ordinary work. The achromatic condensers are corrected for two colors and spherically corrected for two zones. The plane mirror should be used with the condenser. Ordinarily, when using oil-immersion objectives, the condenser is used dry, but the objective is more efficient when there is an immersion contact between condenser and slide also, which is necessary when a dark field condenser is used (§ 174). The best position for proper focusing of the condenser may be obtained by first using a 16-mm. objective; focus upon the object, and adjust the condenser until the image of the window sash or flame is in the same plane as the object.

To determine the relation of aperture of condenser to objective, Bausch (1906) recommends the following: Remove the eyepiece and look through the tube. Close the diaphragm and then gradually open it. When the diaphragm appears to have the same opening as the back of the objective, the condenser has the same angular aperture. For histological objects, the apparent aperture of the diaphragm should be about half the opening of the back lens in the objective.

Dry objectives are corrected for a certain thickness of cover glass, the average for moderate powers being a No. 2 cover, i.e., about 0.18 mm. thick. For work of great refinement, a cover must be employed showing a deviation from the required thickness, for which the objective is corrected, not exceeding 0.03 mm. Several devices for measuring covers are on the market. Slight differences in thickness of cover glass, not exceeding, say, 0.05 mm., may be compensated for by increasing the tube length in case of too thin a cover or shortening it for one too thick. Homogeneous immersion objectives are independent of variations in the thickness of the cover glass, but the correct tube length must be maintained. The finer the definition of the objective, the less range it has in depth of focus. An objective with greater depth of focus than another with the same numerical aperture is not as well corrected.

174. Dark-field Microscopy. For directions for setting-up and manipulation of the apparatus, as well as for an historical account, see Professor Gage's article, " Modern Dark-field Microscopy and

the History of its Development." (Trans. Amer. Micr. Soc. 39 : 95–141, 1920.)

175. Magnification of the Compound Microscope. When one is constantly using a microscope, it is of interest to know the magnifications of the various combinations of oculars and objectives. Magnification tables are published in the catalogues of the dealers in microscopes; and if the microscope and its optical equipment are of the same make, magnifications may be obtained from the tables.

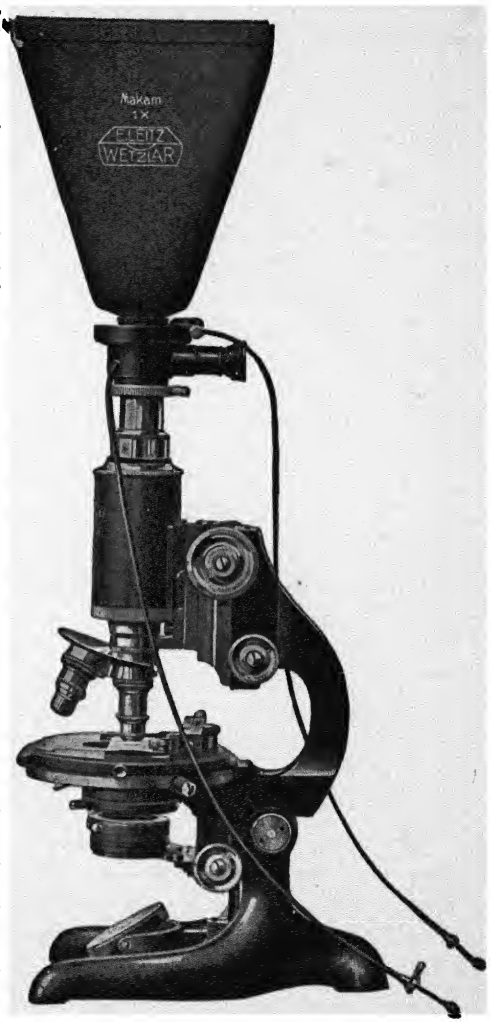


FIG. 9. — Leitz Microscope with "Makam" Camera attached

176. Micrometry. The unit of measure in microscopy is one-thousandth of a millimeter (0.001 mm.), called a micron, and is expressed by the Greek letter mu (μ). A simple way to measure objects under the microscope is to use an ocular micrometer. Adjust the tube to its proper length of 160 mm. Place the ocular micrometer in the eye-piece upon the diaphragm, and a stage micrometer upon the stage of the microscope. Focus, and then determine the number of divisions and the fraction of a division of the stage micrometer which is covered by one division in the

eye-piece micrometer. Thus the value of one division of the eye-piece micrometer is determined for this particular combination of lenses. Repeat for all combinations of eye-pieces and objectives and tabulate the results.

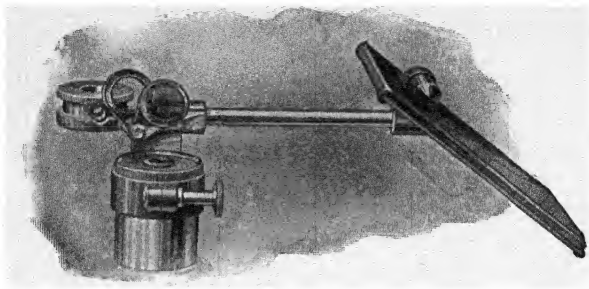


FIG. 10. — Abbe Camera Lucida of Leitz

Suppose one division of the eye-piece micrometer covered 7 divisions of the stage micrometer when using ocular X-10 and a 16-mm. objective, and that these 7 divisions equaled, say, 0.07 mm. with the draw tube at 160 mm. If an object placed on the stage were covered by 9 divisions in the eye-piece with the same combination of lenses, the length of the object would be 9 times 0.07 mm. or 0.63 mm. Measurements may also be made with the camera lucida.

FIG. 11. — Spencer Microscope Lamp

177. Lighting. A north light, especially when reflected from white clouds, is most desirable. If the sun shines in the window, the light may be reduced by passing through a white or bluish screen of some sort. When examining opaque objects, direct or reflected light is needed. This may be direct sunlight or light from some type of lamp. In histology, transmitted light, i.e., light that passes through the object, is nearly always used. The lower the power the less

light is required, the adventitious light being cut off by means of an iris diaphragm. The proper use of the diaphragm will soon be learned by experience. While day-light is preferred by most microscopists, artificial light is often necessary. There are many types of excellent electric lamps to be had, which it will be needless to describe here.

SPECIAL METHODS

THE CELL

The technique of the cell is almost coextensive with that of histology as a whole, at least as far as concerns the application of the more exact and delicate methods. It is necessary, therefore, to give here only the more salient points and accepted methods.

The manner of killing for cytological purposes is apparently not a matter of indifference. McClung recommends the use of xylene for killing Orthoptera. In other cases an insect may be dissected and the gonads or other parts quickly removed in the body fluid or in normal salt or Ringer's solution and then fixed. Some workers decapitate, others kill with cyanide, illuminating gas, or chloroform, the last probably least trustworthy in cytological work. Or the (small) animal may be placed in the hot fixing fluid.

General Methods. Hermann's fluid, Flemming's fluid, Zenker's fluid, mercuric chlorid, Carnoy's fluids, and Bouin's fluid are standard fixers, although special problems may demand other combinations. Iron hematoxylin, the Ehrlich-Biondi-Heidenhain triple mixture, safranin, gentian violet and orange G are perhaps the most serviceable stains. Of these iron hematoxylin is particularly and universally useful. The Ehrlich triple stain is valuable in the more analytical work and should follow mercuric chlorid (or Zenker's fluid, Carnoy's fluid) fixation. Safranin and gentian violet are used separately as red or blue stains, or successively, followed by orange G (Flemming's triple stain) only after Hermann's fluid, or Flemming's fluid, or similar mixtures.

178. Chromatin. While regressive stains of the iron-hematoxylin type give valuable chromatin stains, they are not as a rule analytical or selective. In accordance with the recommendation of Heidenhain, it is better to stain progressively with dilute solutions if a pure chromatin (basichromatin) stain is desired.

Methyl green is one of the most delicate and precise of chromatin stains. It may be used alone or in combination, as in the Ehrlich-Biondi mixture. A very dilute hematoxylin (§§ 90, 93) is excellent.

179. Feulgen Stain. For the use of this sharp and pure chromatin (nuclein) stain, consult Margolena, Stain Technology,

vol. 6, No. 2, 1931, p. 47, and Stain Technology, vol. 7, No. 1, 1932, p. 9.

180. Cytoplasm. Quite different pictures are obtained by the use of fixers as similar in composition as Flemming's fluid and Hermann's fluid, the difference seeming to be due to a varying preservation of cytoplasmic "granules." The groundwork of the cell body, the so-called spongioplasm, may be preserved by such fixers as Zenker's fluid, Flemming's fluid, Hermann's fluid, Carnoy's fluid, etc., and iron hematoxylin and the Ehrlich-Biondi mixture give satisfactory stains. The granules (so-called inclusions) that may be present are of different kinds and often not easily interpreted; they include, (*a*) "reserve" material (yolk granules, fat granules, etc.); (*b*) basophile, acidophile, neutrophile granules; (*c*) granules less easily preserved which include many secretion granules, etc.

(*a*) *Yolk granules.* If present in large amount, a special fixer may be indicated (§§ 24, 25, 35) and a special procedure.

Fat granules; see § 240.

(*b*) *Basophile* granulations (Chromidia, Granoplasma, Unna) may be demonstrated by simple alcohol fixation and subsequent differential staining with basic dyes. Compare § 208 and the special technique of blood (§ 226-).

(*c*) These require special technique: in general, (1) the employment of oxidizers such as dichromates, osmic acid, formalin, (2) no acid, or a minimum amount. This seems to indicate the presence of reducing substances, usually lipoid in nature, whose combinations are soluble in (or rendered soluble by) acid. Here belong the mitochondria (chondriosomes).

181. Mitochondria. Benda's original method (crystal violet and alizarin) is capricious, and, while it is of value, other methods are to be preferred. There may be mentioned:

(*a*) Fixing the tissue with Benda's fluid (§ 34) but employing iron or copper hematoxylin as a stain.

(*b*) Fixing for 12–24 hours in Zenker's fluid or the copper dichromate mixture (§ 16), the acetic acid being reduced to about $_1^1{}_0$ per cent, the chromation being increased by a stay of 3 or 4 days in a $2\frac{1}{2}$ per cent dichromate solution (Müller's fluid or Erlicki's fluid). Paraffin sections (thin) may be stained with iron hematoxylin or copper hematoxylin.

(*c*) *Regaud's method.* Fix in 3 per cent aqueous solution of potassium dichromate 80 parts, formalin 20 parts, for 4 days, changing every day. Continue the chromation by a stay of 7 days in 3 per cent potassium dichromate solution, changing the fluid every other day. Wash in running water for 24 hours. Stain paraffin sections in iron hematoxylin.

(*d*) Fix small pieces in Champy's fluid (§§ 32, 33) or in chrome-osmic fluid for 24 hours. Wash for ½ hour in distilled water, then transfer to a mixture of 1 part acet. pyrolignosum rect. and 2 parts of a 1 per cent chromic acid solution for 20 hours. Wash ½ hour in distilled water and transfer to 3 per cent solution of potassium bichromate for 3 days. Wash under the tap for 24 hours, pass through the grades of alcohol to xylene; imbed in paraffin or paraffin celloidin. Section 4–5 microns.

(1) Stain in Altmann's acid-fuchsin anilin-oil mixture (5–10 grams of acid-fuchsin in 100 cc. of anilin-oil water) and heat until steaming.

(2) Set the slide aside to cool for 6 minutes, pour off and wash in distilled water.

(3) Counter-stain either in 0.5 per cent solution of toluidin blue or in a saturated solution of thionin in distilled water, for 1 or 2 minutes. Wash in distilled water.

(4) Differentiate in a 0.5 per cent solution of aurantia in 70 per cent alcohol for 20–40 seconds, watching the process under the microscope.

(5) Wash in 95 per cent alcohol, then into absolute, xylene, and balsam. The chromatin is blue, mitochondria are red, and the ground cytoplasm is golden-yellowish to green.

The procedure may be modified as follows: Beginning with section 2, setting the slide to cool for 6 minutes, pour off and wash in a solution of picric acid (1 part of a saturated aqueous solution of picric acid diluted with 2 parts of distilled water), heat, blot, dehydrate in 95 per cent and absolute alcohol, then clear in xylene and mount in balsam.

(*e*) *Bensley's method.* Fix the tissue for 24 hours in dichromate-acetic osmic mixture (§ 35). Paraffin sections (thin) are treated for ½ to 1 minute with a 1 per cent aqueous solution of potassium permanganate and then for about the same time with a 5 per cent aqueous solution of oxalic acid. Wash well in distilled water and stain for 5 minutes with a solution of 20 grams of acid fuchsin in

anilin water (i.e., a saturated solution of anilin in distilled water), heating it until it steams. Allow it to cool for about 6 minutes. Rinse away all excess stain with distilled water and stain for a few seconds with a 1 per cent aqueous solution of methyl green. Dehydrate rapidly with absolute alcohol, clear in xylene and mount in balsam. If the acid-fuchsin stain is insufficient, reduce the treatment with the potassium permanganate or restore the chromation by treatment with a $2\frac{1}{2}$ per cent solution of potassium dichromate for about $\frac{1}{2}$ minute; rinse and stain. The treatment with permanganate and oxalic acid may frequently be omitted. Toluidin blue may be substituted for the methyl green.

(*f*) *The Mann-Kopsch method.* Useful for the demonstration of mitochondria, Golgi apparatus (§ 182) and other cytoplasmic constituents. (Hirschler, Arch. f. Mikr. Anat. 89 : 4, 1916; Lee's Vade-mecum, p. 328; Sitzungsber. d. K. Preuss. Akad. d. Wiss., 1902.) Fix small objects, such as the ovary of an insect of moderate size, in Mann's fluid (freshly prepared solution of equal parts of a 1 per cent solution of osmic acid and a saturated solution of corrosive sublimate in normal salt solution) for $\frac{1}{2}$ hour (or longer for denser or larger material), the length of time determined experimentally. Next wash in 2 or 3 changes of distilled water for 20–30 minutes. Transfer to a glass-stoppered container with enough of a 2 per cent solution of osmic acid to cover the material. Leave at room temperature in the dark for about 2 weeks. If the fluid deteriorates, losing the smell or becoming quite black, or if it is evaporating, wash quickly in distilled water and add fresh osmic acid. Wash for 2 hours in running water and dehydrate through alcohols, 50 per cent to absolute. Clear in xylene and imbed in 56–58° C. paraffin. Cut sections 3–7 microns, arranging them on the slide in the usual way. Remove the paraffin from one of the slides with xylene and mount in balsam.

If the Golgi apparatus is black and the mitochondria are yellowish, take another slide prepared as before, carefully treat for a minute or two with $\frac{1}{4}$ per cent of permanganate of potash, wash, and then stain by the Altmann acid-fuchsin-anilin method followed with picric acid differentiation.

If successful, it will yield a beautiful picture. If the mitochondria are blackened, they may be bleached after removal of the paraffin from the sections, by careful treatment with turpentine.

182. Golgi Apparatus. This problematic constituent of the cytoplasm clearly possesses a composition closely resembling that of the chondriosomes (mitochondria) and is demonstrable by similar methods. There may be mentioned as standard: (*a*) the Kopsch (or Mann-Kopsch) osmic acid method (§ 181 *f*), (*b*) the Cajal uranium nitrate and silver nitrate method, (*c*) the DeFano cobalt nitrate method. For the details of their application, consult Gatenby (Lee's Microtomist's Vade-mecum, Chapter XXVI).

THE CHITIN

One of the greatest difficulties encountered in sectioning arthropod tissue is the presence of chitin. It cannot be dissolved without destroying the underlying tissue. It is obvious that the internal organs may be dissected out and cut, but this in some cases results in undesirable distortion and is, with the minuter forms, too difficult. A method which may be successfully used in many cases is to imbed in the usual manner and then, with scalpel or microtome, trim the paraffin block in such a way as to remove the greater part of the chitin and then re-imbed. This, of course, cannot be done when the hypodermal cells lying immediately under the chitin are to be studied. An insect or other arthropod may in most cases be readily sectioned just after molting. It is advisable to adopt this expedient in some cases, but it should always be checked by sections of more mature animals, since the organs and tissues of the latter may differ in essential particulars.

Various methods have been suggested for softening chitin. Some workers maintain that thorough dehydration and de-alcoholizing aids greatly in softening chitin. Where other methods fail, paraffin-celloidin imbedding has given, in the authors' experience, the most uniformly good results in cutting chitin, hence is to be recommended in case difficulties are experienced in getting good sections.

183. Among the earlier recommendations is a thick aqueous solution of soap, to which is added alcohol to make a mixture of the consistency of thin honey. Osterloh (1922) soaked spiders for 3–21 days in it to soften the chitin. Chloroform or cedar oil as a clearer has been found to render chitin less brittle than xylene. Eau de Javelle (hypochlorite of potash) or Eau de Labarraque (hypochlorite of soda) will dissolve chitin in a short time with the

aid of heat. Immersion for 24 or more hours in a dilute solution (15–25 per cent) of either one will soften chitin and has been recommended by some workers. Fixing fluids containing nitric acid, such as Gilson's fluid, are believed to have a softening effect upon chitin. A solution of 70 per cent alcohol containing 5–10 per cent of nitric acid has been used with good effect.

184. Hennings' fluid (§ 21) has been much used for fixing insect tissue. The material should be left in it for 12–24 hours and then washed in 60 per cent iodinized alcohol. The fluid may be used either hot or cold. When the chitin is thin or the animal has recently molted, the nitric acid in the formula may be reduced to 2 parts. Other fluids that have been successfully used for rendering chitin less brittle are Frenzel's fluid (1 drop of nitric acid to 1 or 2 cc. of a half-saturated solution of mercuric chloride in 80 per cent alcohol), Perenyi's fluid, or a warmed mixture of 3 parts of a concentrated aqueous solution of mercuric chloride and 1 part of Perenyi's fluid.

185. When the chitin alone is to be studied, it may be fixed in a fluid made up of 2 or 3 parts of 25 per cent hydrochloric acid, 33 parts of glycerin, and 66 of 80 per cent alcohol. *In toto* preparations may be stained with eosin, carmine, or iodin; sections, with hematoxylin and picric acid.

186. Recently there has been placed upon the market (E. Leitz, Inc., New York) a preparation called Diaphanol. Great claims are made for it for softening and bleaching chitinized structures. Fixed and well-hardened tissues are slit, rinsed in 63 per cent alcohol, and then placed in Diaphanol until they are perfectly bleached and softened, or, in accordance with the experience of an English worker, left in the fluid not exceeding 3 or 4 days. The tissue is best placed in the fluid in glass-stoppered bottles at room temperature. In case of discoloration of the fluid, it must be changed. Replace in 63 per cent alcohol, dehydrate, transfer to tetralin, and thence into paraffin. For the detection of chitin the objects are fully bleached with Diaphanol, then soaked in water and treated with a solution of chlor-zinc-iodin, whereupon the chitin becomes violet.

Botanists in some cases soak paraffin blocks, containing hard plant structures, for weeks in water, with the result that sections may be cut much more readily. This method has had a limited trial for chitinized structures in the authors' laboratory. The

results are encouraging enough to warrant further experimentation.

187. Staining Chitin. Arthropods that have been treated with caustic potash may be stained in an alcoholic or glycerin solution of pyrogallic acid. If too dark, they may be bleached in a weak acid. Prell (Zoölogica, 64 : 13, 1913) states that preparations that have been treated with caustic potash may be stained with a $\frac{1}{2}$ per cent solution of water blue in 95 per cent alcohol. Silver nitrate impregnation methods are complicated and not permanent.

Preparations treated with caustic potash may also be stained with acid fuchsin, $\frac{1}{4}$ per cent aqueous solution of picric acid, or gentian violet. It is imperative that the caustic potash be thoroughly washed out. Treatment with acidulated alcohol before dehydration is recommended. Chitin in sections is rather difficult to stain in some cases, owing to the difference in the nature of the chitin in some species. Hematoxylin and acid-anilin dyes will give good results in some instances.

Ziehl's carbolic fuchsin also gives good results. Fuchsin, 1 gram; crystals of carbolic acid, 5 grams, 95 per cent alcohol 10 cc.; distilled water, 100 cc. Differentiate with alcohol followed by clove-oil.

Mallory's anilin-blue connective-tissue stain (§ 121) may be used to advantage in staining chitin in thin sections. The tissue is fixed in Bouin's or Zenker's fluid. The differentiation may be watched under the microscope. The primary cuticula is stained red, and the secondary cuticula a clear blue.

CONNECTIVE TISSUE

188. White (Collagenous) Fibers. Fuchsin acid is particularly valuable. Three methods of applying it for the differential staining of connective tissue follow:

(*a*) *Picro-fuchsin.* See § 120. This may be used with or without a basic counter stain, which should precede it. If a counter stain is used, remember to over-stain and use the picro-fuchsin to differentiate it.

(*b*) *Orange-fuchsin. Formula:* Fuchsin acid, 2 grams; orange G, 1 gram; glycerin, 7 cc.; distilled water, 100 cc. Fix tissue in Flemming's fluid. Stain sections 30 seconds; dehydrate, clear, and mount in balsam (not alkaline). Suitable also for staining the reticular tissue (lymphatic tissue).

Somewhat more delicate than the picro-fuchsin.

(*c*) Mallory's connective-tissue stain. See § 121. While not a differential stain for collagenous fibers, it is nevertheless a valuable one, and frequently to be preferred.

189. Elastic Fibers (Elastin). Employ either the Weigert resorcin-fuchsin or the orcein methods (§§ 126, 127), or the Verhoeff [44] method (§ 128).

Both the white and the elastic fibers may be stained in the same preparation, the elastic fibers being stained first.

In a mixture composed of white and elastic fibers, picro-fuchsin (§ 120) will stain the elastic fibers a light yellow, the white fibers being colored red.

In studying the connective tissues, it should be remembered that acetic and the mineral acids cause swelling or solution (gelatinization) of the white fibers, depending upon their strength. While this improves the cutting quality of organs rich in connective tissue, it also causes vagueness in outline of the white connective-tissue fibrils. It may therefore be advisable to decrease the percentage of acetic acid in the fixer when the connective tissue is under investigation.

190. Reticular Tissue. Fuchsin acid, Mallory's connective-tissue stain, or the orange-fuchsin acid mixture may be chosen for staining this form of connective tissue. As, however, the cellular elements usually mask the fiber relations, if a view of the latter is desired the cells must be removed, by a mechanical method, such as cautiously brushing the section with a camel's hair brush, or by digestion.

191. Digestion Method. The organ to be examined (e.g., lymphatic node, spleen, etc.), is preserved in 67 per cent alcohol, cut into sections 2 mm. or more in thickness, washed thoroughly in water to remove the alcohol, and digested with pancreatic solution in 1 per cent sodium carbonate solution in an incubator at 38° C. until examination under the microscope shows that the cells have become disintegrated and digested. The digestion fluid should be changed every day or every 2 or 3 days. A varying length of time is necessary, — sometimes a month or more. Paraffin sections may also be submitted to digestion, with or without the removal of the paraffin.

The following method is recommended for the further preparation of digested tissue. After washing thoroughly in running water to remove the digestion fluid, the tissue is carefully imbedded in celloidin (§ 61–) and sectioned, the sections being 20–30 microns thick. These sections are not fastened to the slide, but after having been brought into 95 per cent alcohol are placed in a

concentrated solution of acid fuchsin in 95 per cent alcohol to which a drop or two (per 50 cc.) of glacial acetic acid has been added. After several minutes, the sections are rinsed in 95 per cent alcohol to remove the excess stain, cleared in carbol-xylene and mounted in balsam. By this method the delicate morphology is preserved and a sharp stain is secured.

The method of artificial digestion with trypsin or pepsin has other applications in the histological analysis and it possesses a distinct value.

CALCIFIED STRUCTURES — BONE AND TEETH

Decalcification

192. For the purpose of investigating the soft structures of tissues containing lime salts, such as bone, teeth, and calcified cartilage, it is necessary to remove the lime salts before sections can be prepared in the usual way, by a process known as *decalcification*. Solutions of a large number of acids, combined or uncombined with other substances, may be used as decalcifiers. Very satisfactory are:

(1) hydrochloric acid, 1 cc.; 67 per cent alcohol, 100 cc.

(2) nitric acid, 3 cc.; 70 per cent (67 per cent) alcohol, 97 cc., and

(3) nitric acid, 5 cc.; saturated aqueous solution of (potash) alum, 50 cc.; water, 50 cc.

In the first and second formulas the alcohol acts as a restrainer of the acid, while in the third the alum performs this office. The first or second of these formulas is, perhaps, better for bone; the second has a more rapid action and is possibly a better decalcifier for teeth. It is better to let the decalcification proceed slowly for a longer time in an abundance of fluid changed often, in order that the carbon dioxid may not be formed too fast, accumulate in the tissues, inflate and distort them. Many fixers contain acid, and in their action give decalcification enough in the case of small calcified objects. Gilson's fluid (§ 14) is particularly useful.

193. Directions for Use. [14.] The tissue to be decalcified had best be first thoroughly fixed and hardened by one of the approved methods, and should be in 82 per cent alcohol. In fixing, structures not needed should be removed, — muscles trimmed away from the bone, etc. Bones or teeth should be opened with

nippers or a saw, so that the fluid may reach the marrow or pulp cavity.

Place the hardened tissue in the decalcifier, where it should remain until the lime salts have been entirely removed, as may be ascertained by inserting a fine needle; if any calcified matter remains there will be a gritty feeling on using the needle. The time necessary for complete decalcification will depend upon the size and density of the calcified tissue, and will vary from 3–15 days or longer. The decalcifier should be changed after the first day, and if the tissue is large it is best to change it subsequently, two, three or more times at intervals of several days.

When decalcification is complete, rinse the tissue well in water or 67 per cent alcohol for a few minutes and place it in 67 per cent alcohol for one or two days and then in 82 per cent alcohol for several days, or until ready to imbed. The 82 per cent alcohol should be changed once or twice in order that the nitric acid may be well washed out. Although paraffin in many cases may be employed for imbedding, the celloidin method is generally more satisfactory.

Hematoxylin with eosin, hematoxylin with picro-fuchsin, and hematoxylin with eosin and orange or erythrosin and orange, afford good stains; by staining thoroughly with hematoxylin a differential staining of bone and cartilage may be obtained. Mallory's connective-tissue stain frequently gives interesting pictures.

Sections of Dry Bone or Tooth

194. Though the general structure of bone and tooth is shown moderately well when the tissue has been decalcified (§ 192), the Haversian canals, canaliculi and lacunæ of bone and the dentinal tubules of the teeth are shown much better in sections of dried, non-decalcified, tissue, rendered sufficiently thin for microscopic examination by grinding or filing.

195. Directions for Procedure. Prepare thin transverse sections of dried bone in accordance with the directions below. Longitudinal (radial) sections and tangential (surface) sections may also be prepared in the same manner, the former to show the Haversian canals and their anastomoses, the latter to indicate the shape of the lacunæ as seen in a different plane.

(1) *Sawing the section.* Make an exact transection of a part of the shaft of a long bone. The section should be about 1 cm. long

and should include the thickness of the shaft from the surface to the medullary cavity. Make the sections about 1 mm. thick.

(2) *Grinding the sections.* Place the piece of bone on a cork or piece of soft wood and wet it with water. File it on one side until smooth and then turn it over. Continue the filing till the piece is 0.05–0.10 mm. thick, using the cover-glass measurer to determine the thickness. In the beginning one can press quite hard in filing; as the section thins, more care should be exercised and the pressure should lessen.

A grinder, such as a fine carborundum wheel or emery wheel connected with a variable-speed electric motor, is very useful and greatly expedites the preparation of the sections. The carborundum wheel should be horizontal and the sections ground on the flat surface of the wheel, water being used to carry away the bone dust.

(3) *Washing and drying the section.* When the section is thin enough, rinse it and dry it with lens paper.

(4) *Mounting the sections in hard balsam.* To prepare the balsam, put 2 or 3 large drops on the middle of a slide and heat the slide in some way to drive off the volatile constituents. Do not heat the balsam hot enough to produce bubbles. When the balsam chips after cooling, it is ready for use.

In mounting, have the section and a clean cover so placed that they may be easily and quickly grasped. A cork somewhat smaller than the cover glass should be within reach, and also a stone or piece of glass upon which to cool the specimen quickly as soon as it is mounted.

Heat the slide until the balsam is well melted. Put the slide upon a piece of paper, grasp the piece of bone with the forceps, and plunge it into the melted balsam; put on the cover as quickly as possible and press it down with the cork; finally put the slide on the stone or glass to cool the balsam quickly. All of this should be done as rapidly as possible, and, if done rapidly, the air will be retained in the lacunæ and canaliculi, and cause them to stand out as black spots and lines. If soft balsam were used it would soon drive out the air, and, being of nearly the refractive index of bone, it would obliterate the lacunæ and canaliculi. Further, if the hot balsam were not *cooled quickly*, the air would be driven out and balsam would take its place in the spaces.

196. Calcified structures of invertebrate animals may usually be prepared in either of the above ways.

DESILICATION

197. When it is necessary to remove the silicates from animals possessing a siliceous skeleton, hydrofluoric acid must be employed. Care must be taken to avoid breathing the fumes, and the process of silication must be carried out in dishes coated with paraffin.

To tissue in alcohol, the acid is added drop by drop. A weak solution usually suffices, and 24 hours' action may be taken as a maximum.

DEPIGMENTATION

198. Grenacher's solution for removing the color from the pigment cells in arthropod eyes is made up of 50 parts of glycerin, 50 parts of 70 per cent alcohol, and 2 or 3 parts of nitric acid. A modification used by Hennings is composed of 65 parts of 80 per cent alcohol, 33 parts of glycerin, and 2 parts of concentrated nitric acid. The eyes of myriopods may remain in the solution for 12 hours at 35° C. The eyes of some insects require less time. For lepidopterous eyes, Eltringham substituted hydrochloric acid for the nitric acid in Hennings' mixture. Diaphanol may be used as a depigmenting agent.

MUSCLE

199. Fresh. Much of the investigational work on muscle has been done on fresh muscle or frozen sections. For the examination of fresh muscles, it is advantageous to have them very thin. Of the several muscles that have been recommended, one of the most available is the M. cutaneus pectoris of the frog. This may be prepared by cutting the skin in the midventral line, cutting at right angles to the first cut across to the angle of the jaw, thence caudally parallel to the first cut. The skin flap so formed contains the insertion of the muscle which may now be easily dissected free and removed together with some of the tissue at insertion and origin to handle it by. It may be used for examination while fresh, with the polarization microscope, etc.

200. Isolation Methods. Nitric acid (§ 45) may be used for plain muscle and for skeletal muscle. Potassium hydroxid (§ 46) is suitable for heart muscle.

Nitric Acid. Place in the nitric acid dissociator the fresh striated muscle, gland, or organ containing the muscle (plain

or striated) that it is desired to isolate. If it is the intention to work out the anatomy of the muscle or the relation of the muscular coats in an organ, the entire muscle or organ should be taken; otherwise, portions will suffice. At the ordinary temperature of the laboratory, the dissociating action will have been sufficient in 1–3 days; test at intervals with needles to ascertain whether the fascicles and fibers can be easily separated; or fragments may be shaken in a test tube or vial with water in order to separate the fibers.

When the dissociation is sufficient pour off the acid and wash the muscle gently but thoroughly with water. If the tissue is to be stained with hematoxylin or carmine, or kept for any length of time, drain off the water and add a half-saturated solution of alum. For permanent storage, pour off the alum solution and place successively in 67 per cent and 82 per cent alcohol.

For *temporary examination*, tease out a portion of the muscle in water, separating the fibers carefully by means of needles; cover and examine.

Permanent preparations. (*a*) *Unstained.* After teasing out with the needles, drain off the water and add a small drop of glycerin or glycerin jelly; cover, and seal after first properly cleaning (§§ 164, 165). (*b*) *Stained.* Either employ the nitric acid method given above or (better, W. M. Rogers) place the muscle in a mixture of saturated solution of picric acid 8 parts, glacial acetic acid 2 parts, in which it remains until dissociation is complete. Test at intervals, and when dissociation has proceeded far enough, wash out the dissociator thoroughly with 67 per cent alcohol. Stain with carmine or preferably hematoxylin. Mount in glycerin (§ 155–), glycerin jelly (§ 158), or dehydrate, clear and place in dilute balsam (§ 159–, Cf. § 230). The last is to be preferred.

201. Sections. To bring out the structure of the fibrillæ, picro-aceto-formol (§ 19) or an alcoholic fixer (§ 26) is preferable, although the sarcoplasm is not so well preserved. The muscle should be moderately distended upon cork, before fixing, the ends secured by small pins. Ten per cent formalin, Zenker's fluid, or mercuric chlorid may also be used, the sarcoplasm being much better fixed in dichromate, osmic acid, or formalin mixtures. Iron hematoxylin is particularly indicated as a stain for muscle (§ 95). Mallory's connective-tissue stain (§ 121) is found to give an excellent differentiation (Kingery).

To differentiate muscle *in situ*, picro-fuchsin may be used; this stains muscle yellow or orange, the surrounding connective tissue red. Mallory's connective-tissue stain is also frequently useful.

202. To differentiate the intercalated discs of cardiac muscle, fix in a mixture of absolute alcohol 90 parts and 25 per cent nitric acid 10 parts for 24 hours. Wash in several changes of 95 per cent alcohol to remove the acid. Imbed in paraffin. Stain sections in hemalum (§ 91) of $\frac{1}{10}$ strength 12–24 hours. Differentiate with acid alcohol if necessary; dehydrate, clear, mount in balsam. The staining may be given *in toto* before the imbedding, in which case, stain several days. The above is Zimmermann's method. Fixation in mercuric chlorid (§ 11), Zenker's fluid (§ 12) or Carnoy's fluid with subsequent staining of sections in vanadium hematoxylin, phospho-tungstic hematoxylin, or iron hematoxylin, respectively, has also been employed successfully in the study of heart muscle, and for the differentiation of the intercalated discs.

203. Silver methods (§ 211), such as the following, are frequently useful. Small pieces of muscle fiber of invertebrates, not exceeding 4 mm. in thickness, may be fixed in a mixture of 15 parts of a 1 per cent solution of uranium nitrate and 100 parts of formalin for 8–12 hours. Quickly wash in distilled water, transfer to a 1 per cent solution of silver nitrate for 24 hours, then, after washing again in distilled water, place in a reducing solution (hydrochinon, 2 grams; formalin, 6 cc.; distilled water, 100 cc., to which is added a minute quantity of Glauber's salt) for 24 hours. Then, after washing again in distilled water, dehydrate, clear, and imbed as usual.

THE NERVOUS SYSTEM

An analytical grouping of the numerous methods used in the study of the finer structure of the central nervous system is premature. The most salient point is the prominent part that reduction processes seem to play. The more important methods here presented deal with (*a*) the finer structure of cell and fiber — demonstration of the tigroid substance and fibrillæ; (*b*) the differential staining of the myelinic nerve fiber (Weigert and Marchi methods); (*c*) the morphology of the elements (neurones) as revealed by the chrome-silver impregnation methods (Golgi methods) or the use of intravitam (methylene blue) methods.

204. Isolation of Nerve Cells. Employ formaldehyde dissociator for the isolation of the nerve cells of the spinal cord and of the cerebral cortex, proceeding as follows:

Split the spinal cord along its median plane, separating thus the two halves, and place it in an abundance of the dissociating fluid. The cerebral cortex should be cut into small pieces by sections vertical to the surface. Allow it to remain in the dissociator 2–24 hours; for the best results, a stay in the fluid of more than 24 hours is not so satisfactory; although isolated cells are readily obtained, their processes are broken off much nearer the cell body.

Place a fragment of the gray matter of the spinal cord or the cortex of the cerebrum on a clean slide in a drop of $\frac{1}{4}$ per cent Congo red (§ 114) or $\frac{1}{10}$ per cent eosin in formaldehyde dissociator; with the blade of a scalpel, crush the tissue, grinding it thoroughly with a rotary movement, which will reduce it to small pieces. Gather the débris, drain off the fluid, and add a drop of glycerin containing stain. Cover and examine, tapping the cover sharply with the handle of the scalpel to shake out the processes of the cells and free them from surrounding matter. Examine, searching for cells with many and long processes, and, if a satisfactory preparation has been obtained, seal according to § 164.

205. Isolation of Nerve Fibers. (*a*) For isolation of myelinic nerve fibers, with the preservation and blackening of the myelin, and for amyelinic fibers, employ osmic acid dissociator (§ 43).

(*b*) For the isolation of myelinic nerve fibers with the removal of the myelin for the demonstration of the axis cylinder, neurolemma, and framework of the sheath, fix nerves in dichromate-acetic or similar fluid for one or more days, wash in water, pass up through the alcohols, dehydrate, remove the myelin by placing the tissue in a fat solvent, — chloroform, for one or more days, 95 per cent alcohol 1 day, pass through the alcohols to water, stain in Delafield's hematoxylin 12 to 24 hours, wash in water, pass up through the alcohols, dehydrate, and place in clearer. Out of this the small bundles of the nerve fibers may be teased apart with needles, care being taken to keep the fibers as nearly parallel as possible. Mount in balsam.

206. Gold-chlorid Methods. These methods, which are widely serviceable, depend upon the reduction of gold-chlorid solutions by the tissues through the agency of (*a*) sunlight or (*b*) various chemical substances of which the acids, formic, acetic, citric, etc.,

are particularly used. Either one or both of these agencies may be used. Usually, fresh tissue is used although the method may be applied to fixed tissue, particularly as a neurofibrilla stain [10, 33]. It is the method *par excellence* for staining motor-nerve terminations for which purpose the following method is serviceable.

207. [23.] Fresh tissue or (better) tissue fixed in 10 per cent formalin may be used. Place small pieces of muscle containing the endings for 30 minutes in 10 per cent formic acid solution. Remove to 1 per cent gold chlorid for 30–40 minutes, avoiding direct sunlight; the tissue becomes yellow. Transfer again to a 2 per cent formic acid solution, in which the tissue should remain for 1 or 2 days in the dark (rich purple color). A bluish-purple indicates too great a reduction. Wash in distilled water for an hour or so.

If it is desired to make a teased preparation, transfer to glycerin in which the fibers may be cautiously teased apart, taking care not to separate them too much. Permanent mounts may be made in glycerin or glycerin jelly.

If sections are called for, dehydrate, clear (xylene method, § 51), and imbed in paraffin. Celloidin may also be used.

With fresh tissue the treatment by formic acid may cause too marked a swelling and distortion; in which case the Ranvier method [33] may preferably be used.

208. Nissl Substance (Nissl's Bodies). The stainable substance in the cell body of nerve cells resembles the chromatin of the nucleus in its reactions, staining with basic stains, of which a number are suitable. Alcohol, Carnoy's fluid (§ 29, 30), mercuric chlorid, or formalin may be used as fixers, preferably one of the first three. Methylene blue or toluidin blue is the usual stain.

209. Nissl's Method (*modified*). Imbed in paraffin or celloidin tissue that has been fixed in 95 per cent alcohol or Carnoy's fluid; cut the sections rather thick, 15 to 20 microns. The sections may either be fastened to the slide or carried on as free sections. Stain the sections in a 1 per cent aqueous solution of methylene blue or toluidin blue for 5–10 minutes, heating it until it steams. Permit it to cool, rinse in water, dehydrate, and differentiate in absolute alcohol, clear with oil of cajuput and xylene, and mount in xylene balsam. The nerve cells and nuclei will be stained blue, all else colorless. In the cell bodies of the nerve cells, the tigroid substance will be stained. Should the stain not be selective enough, differentiate for a few seconds before dehydrating with a mixture of anilin 1 part, 95 per cent alcohol 9 parts.

If celloidin is used for imbedding, it should be remembered that it should be dissolved away in the differentiation (absolute alcohol).

210. Held's Method (*modified*). Fix in mercuric chlorid (or as for Nissl's method), imbed in paraffin, cut sections 5 to 10 microns, and stain 15 minutes in 1 per cent solution of erythrosin in 67 per cent alcohol, rinse, stain 10 minutes in 1 per cent aqueous solution of toluidin blue, differentiate briefly in $\frac{1}{10}$ per cent alum solution, dehydrate rapidly in 95 per cent and absolute alcohol, clear in xylene, mount in balsam. Alkaline methylene blue or Nissl's soap solution ($\frac{1}{2}$ per cent methylene blue in $\frac{1}{8}$ per cent soap solution) may be used in either of these two methods, if desired.

211. Silver Methods (Simarro-Cajal Methods). Two of Cajal's methods may be given. *Formula 3a:* (1) Fixation in ammoniacal alcohol (2–10, usually 4–5, drops of ammonia per 50 cc. of 95 per cent alcohol), 20–48 hours; (2) mop up with absorbent paper; and (3) place in $1\frac{1}{2}$ per cent silver nitrate solution for 4–5 days at 32–40° C.; the tissue when ripe should be light gray; (4) wash for a few minutes in distilled water; (5) reduce in a solution of 1–2 grams hydrochinon or pyrogallol; water, 100 cc.; formalin, 5 cc.; for 24 hours; (6) wash in water; (7) imbed by the paraffin method; (8) section and mount in balsam or damar. Recommended for spinal cord, cerebellum, spinal ganglia.

Formula 4a: (1) Fix small pieces of tissue (5 mm. thick or less) for 6 to 12 hours in formalin 15 cc., water 85 cc.; (2) wash 6 hours or longer in running water; (3) place for 24 hours in 50 cc. of alcohol with 5 drops of ammonia added; (4) mop with absorbent paper; (5) place in $1\frac{1}{2}$ per cent silver nitrate solution for 4–5 days (35–38° C.). The remaining steps as in Formula 3*a*. Recommended for sympathetic ganglia, cerebrum, cerebellum.

212. The following two methods are useful: (*a*) (Krause; Bielschowsky, modified). (1) Preserve the tissue in 10 per cent formalin; (2) wash in distilled water 24 hours; (3) 2 per cent silver nitrate, 6–8 days; (4) rinse for a short time in distilled water; (5) to 20 cc. of 2 per cent silver nitrate add 3 drops of 40 per cent sodium hydroxid solution and dissolve the precipitate that forms by adding ammonia drop by drop; in this let the tissue remain 12–24 hours; (6) rinse in water; (7) place in 20 per cent formalin for reduction (about 24 hours). Paraffin imbedding. Cut sections 15–20 microns. Useful for the morphology of ganglion cells as well as cells of the central nervous system.

(*b*) (Ranson's pyridine method). (1) Place tissue in absolute

alcohol with 1 per cent of ammonia added, for 48 hours; (2) wash ½–3 minutes in distilled water; (3) place in pyridine for 24 hours; (4) wash well in distilled water for 24 hours, changing it frequently; (5) 2 per cent silver nitrate (in the dark) for 3 days; (6) rinse in distilled water; (7) 4 per cent pyrogallic acid in 5 per cent formalin for 1 or 2 days. Paraffin imbedding. Designed for the demonstration of amyelinic nerve fibers but useful for ganglia as well.

By these " photographic " methods, fibrillæ, fibrillar networks, changes in histogenesis, etc., may be demonstrated.

For other methods of demonstrating the neurofibrillæ, — Bielschowsky's, Bethe's toluidin-blue method, Apáthy's hematein method, etc., consult larger works on technique [10, 33].

213. The Weigert Method for Staining Differentially the Myelin of Myelinic Nerve Fibers. This method, in all its various forms, depends upon the power of the myelin, probably through the reducing fatty acid present, to combine with and hold in (nearly) insoluble form the chromium (oxid), which thus serves as a primary mordant for a copper or iron hematoxylin stain, which is subsequently differentiated by an oxidizer as a bleacher. The important steps are: (1) Fixing and mordanting in dichromate solutions; usually the potassium salt is chosen; (2) a second mordantage in copper (acetate); (3) the staining; (4) the differentiation. The point at which the imbedding and sectioning are introduced is of secondary importance. It should be remembered, however, that the fatty substances (lipoids) of the myelin upon which the method depends are soluble in the reagents of both the paraffin and celloidin methods, less so in the latter — and in acids, and that even the dichromate mordantage does not preserve them perfectly. The dichromate mordantage must thus be given before the imbedding is begun (alcohols), and preferably with the fresh tissue. Practically the only fixer that is indifferent in this respect, and after which the dichromate may first be used, is formalin. Other dichromate fixers, such as Zenker's fluid with the acetic acid reduced to about ⅕ per cent, may be used, but it is well to let them act only a relatively short time, and continue the mordantage with simple dichromate solutions. Aside from this, the point at which the imbedding and sectioning are introduced is of secondary importance; thus, Strong [50] combines the copper and chromium mordantage by using copper dichromate; the former may also follow the dichromate treatment before imbedding is begun; in

case of celloidin imbedding it may be applied to the celloidin imbedded block, or after the sections are cut. Streeter [49] stains (*in toto*) 4–6 days as well as mordants before the imbedding. Weigert has added to both the primary and secondary mordants chromium fluorid to (1) hasten the process and (2) prevent precipitates; the formulas are: (*a*) 5 per cent potassium dichromate, 100 cc.; chromium fluorid, 2 grams; (*b*) 5 per cent copper acetate, 100 cc.; chromium fluorid, 2 grams; glacial acetic acid, 4 cc. (*b*) is especially indicated if the secondary mordantage is given before the sectioning. Whatever modification of the method is employed, the reduction of the dichromate by the tissue in the primary mordantage should fully reach the dark-brown stage, but not pass it (i.e., become green). Sheldon [47] gives a good résumé of the method. The following method is serviceable:

(1) Fix tissue for 1–2 days in Zenker's (see above), Orth's, or Helly's fluid, 10 per cent formalin, Müller's fluid, or potassium dichromate solution.

(2) Mordant until dark brown in 3 per cent and 5 per cent aqueous solution of potassium dichromate. This usually takes about 4 weeks; or, shorten the period by using the dichromate-chromium fluorid mixture (above) when about 5 days should suffice;

(3) Wash in running water for 1 or 2 days, and

(4) Pass up through the alcohols, preferably keeping the tissue in the dark.

(5) Imbed in cellodin or paraffin.

(6) Stain and differentiate sections by the Weigert copper hematoxylin method (§ 97).

(7) Mount in neutral or alkaline balsam.

Large sections are usually best carried on as free sections. The differentiation of the stain should be carefully watched and stopped when the fibers are a rich dark blue on a yellow-brown background. The reagents used in dehydrating, clearing, and mounting should be neutral or alkaline, — *not acid.*

214. Pal's method may be used if it is desired to stain the nerve cells subsequently.

Fix and mordant in the dichromate, as above; omit the copper mordantage; imbed and section, staining the sections with the strong hematoxylin used in the Weigert method (§ 213) until the sections are a blue-black.

Rinse the sections in tap water and *differentiate* by treating for a *short time* (20–30 seconds) with a $\frac{1}{10}$ per cent aqueous solution of potassium permanganate, and for a few seconds with a mixture of 1 per cent oxalic acid and 1 per cent potassium sulphite, equal parts. The action will be very rapid and must be carefully watched. Wash the sections for $\frac{1}{2}$ hour in running water. Counter-stain with a red stain (eosin, erythrosin, carmine, etc.) if desired.

215. Marchi Method. This method of staining differentially degenerating myelinic nerve fibers depends upon the fact that potassium dichromate (or chromic acid) is able to satisfy the reducing power of myelin but does not oxidize the globules of free fatty acid (?) formed in the degeneration of the myelinic sheath of the fiber, which may be subsequently blackened by the reduction of osmium tetroxid. Important points in the successful application of the method are: (*a*) the length of time the degeneration should be allowed to proceed before treating with potassium dichromate, (*b*) the time in the dichromate mordant, (*c*) the time in the osmic acid mixture (sufficient and complete penetration), and (*d*) the preservation *in situ* and final mounting of the osmicated fat granules. The difficulties here are those of fat preservation in general (§ 240).

(*a*) The optimum will vary and must often be determined experimentally: in general — for cold-blooded animals (toad), 30–40 days; for mammals, 12 days.

(*b*) Eight to 10 days in Müller's fluid or 3 per cent potassium dichromate is usually enough; a longer time does no harm.

(*c*) The osmic acid is useful in 1 per cent aqueous solution, usually mixed with a potassium dichromate solution. Six to 10 days suffice; a longer time does no harm (brittleness).

(*d*) See below.

216. As Employed by Van Gehuchten:

(1) Harden in 3 per cent potassium dichromate solution for 3 weeks;

(2) Transfer to a mixture of 1 per cent osmic acid solution, 1 part; 3 per cent potassium dichromate, 4 parts; for 3 weeks, blocks of tissue not more than 2 mm. thick. Use abundance of the fluid and change 2–3 times if deemed necessary.

(3) Wash in running water for 12–24 hours.

Avoid the paraffin and celloidin imbedding methods if possible.

Of these two, the celloidin method is preferable. For further treatment, see § 243.

217. Flemming's or Benda's fluids (§§ 34, 243) may be used instead of the Marchi method, for the same purpose, with small objects, and peripheral nerves, etc.

218. The Golgi Methods, whose field of application is not confined to the nervous system (gland ducts, bile capillaries, blood capillaries, secretory canaliculi, muscle, etc.) consist in (*a*) *mordanting* the fresh or living tissue for a sufficient length of time in a *dichromate* solution, usually containing, as well, osmic acid or formalin, and then — (*b*) transferring to a *silver nitrate* solution, whereupon certain of the nervous elements become outlined more or less completely by impregnation with a chrome-silver combination.

The reaction probably depends on the presence, in certain "physiological states" of the elements of a substance or substances which combine with the chromium salt (with reduction?) and through it with the silver salt. These hypothetical substances — or possibly physical states — seem to disappear more or less rapidly after the death of the animal, and their power to hold the silver in combination seems to decrease with the progress of the dichromate mordantage beyond a certain point. If successful, certain of the cells and their processes — amyelinic, and to a certain extent, myelinic nerve fibers — are outlined by an impregnation, black by transmitted, brown by reflected, light.

219. The method is, however, capricious; success depends on (*a*) the *kind* of animal; different parts and tissues react more satisfactorily in some animals or classes of animals than in others. (*b*) The *age* of the animal; some regions of the nervous system give better results in young or fetal animals; other parts take the stain better in older animals, etc.; (*c*) the *time* of mordantage; it is necessary that the tissue be mordanted a certain length of time, constant (relatively) for a certain kind of tissue under the conditions above (*a* and *b*). It is necessary that the best amount of dichromate mordantage be given. (*d*) Different organs and regions of the central nervous system vary greatly in the ease with which they can be made to furnish satisfactory impregnations. Almost certain impregnations of hippocamp can be gained; cerebral cortex is likewise quite easy to stain. With the olfactory bulb the action is not constant though fairly complete. The optic lobes

and retina of birds and large reptiles are more satisfactory than those of mammals. The myel (spinal cord) of embryo birds (7–14 day chick best) is generally more satisfactory than that of mammals; in any case, fetal or new-born animals should be employed. It is difficult to make satisfactory impregnations of sympathetic ganglia, organs of special sense, and the intrinsic nerves of the viscera.

The important forms of the method are: (*a*) the *slow method;* mordantage in dichromate solutions of preferably increasing strength, 2, 3, 5 per cent, for 1–4 months depending on the temperature, strength of solution, etc.; (*b*) the *rapid method;* in this another oxidizer, osmic acid, is combined with the dichromate with a reduction in the duration of the treatment to a few days. Combination of (*a*) and (*b*) is sometimes serviceable; (*c*) *double* (or triple) *impregnations*, obtained by repeating (*b*). Important modifications are: (1) substitution of formalin for the osmic acid in (*b*), (2) mercuric chlorid instead of silver nitrate (Cox's method). The Golgi methods have been widely applied and for details the individual papers may be consulted [10].

220. Golgi's Rapid Method. This is the most generally serviceable of the different methods.

Directions for use. Tissue of a (preferably) young animal is placed in a mixture of 4 parts of 3 per cent potassium dichromate and 1 part of 1 per cent osmic acid. The amount of the fluid should be at least twenty times the bulk of the tissue and should be changed as soon as it grows turbid or loses the strong characteristic odor of the osmic acid.

After the action has proceeded to the right degree (§ 221), rinse the tissue in water for about 5 minutes and place for 15 minutes in a $\frac{1}{4}$ per cent solution of silver nitrate, and then for 2 or more days in a $\frac{3}{4}$ per cent solution of silver nitrate, preferably keeping it in the dark.

Without washing, imbed rapidly in celloidin as follows:

(*a*) *Dehydrate* for 2–3 hours in 95 per cent alcohol, changed two or three times; (*b*) place in thin celloidin for 20 minutes, in thick celloidin for 20–30 minutes; (*c*) imbed in thick celloidin, on a block of wood (best; § 65*a*); (*d*) harden the mass in chloroform for 20–30 minutes; and (*e*) place the block in clarifier and cut, sections being 50–100 microns thick according to the nature of the tissue and the character of the impregnation; (*f*) place the sections in

95 per cent alcohol for a few minutes; clear in carbol-xylene and mount in balsam by placing the section on the slide, absorbing the clearer *thoroughly* by means of tissue paper and spreading over it thick xylene balsam. Do not cover. Later, when the balsam has hardened somewhat, it may be melted by heat and much of the superfluous balsam drained from the section and scraped away with a knife, and a cover glass added if desired.

221. *Time of hardening.* From results of Cajal, van Gehuchten, and others, and from general laboratory experience, the following periods will probably be found approximately correct. In general: The best results are to be obtained with kittens 3-20 days old, puppies 2 weeks old, rats 8–10 days, rabbits 8 days. (*a*) For cerebral cortex (and hippocamp): New-born kitten, 1–2 days; kitten half grown (3–4 months), 3–4 days; new-born rabbit, 24 hours; rabbit 1 month old, 2–3 days; adult mice, 3–4 days.

(*b*) For spinal cord: Chick of 5–6 days' incubation, 24 hours; chick, 14–15 days' incubation, 3 days; new-born kitten or puppy, 2–3 days. Frog tadpoles (large) 3–5 days.

(*c*) Cerebellum: New-born kitten, 1–2 days; kitten half grown, 4 days.

(*d*) Sympathetic system: Chick of 14–18 days' incubation, 3 days.

(*e*) Retina: 1–3 days.

(*f*) Olfactory mucous membrane: 3–4–7 days.

222. Golgi's rapid method may be used for demonstrating the finer structures of the olfactory organs on the antennæ of bees and wasps. After treatment in this manner the material may be cleared in chloroform and imbedded in paraffin.

223. Intra Vitam Methylene Blue. Methylene blue shares with a number of coal-tar dyes the power of staining during life nerve cells and fibers and certain cytoplasmic granules. Like the Golgi methods, " intra vitam " staining of nervous tissue is capricious; it is applicable only to living or fresh tissue and depends upon unknown substances or conditions that become changed after death. In the reaction, reduction of the methylene blue to its leucobase (colorless) by the nervous tissues appears to play an important part. In general, the technique involves: (1) Bringing a methylene blue solution of sufficient strength in contact with the (essentially) living nervous elements; (2) permitting it to remain a sufficient length of time for the staining reaction; (3) exposing

the tissue to the action of the oxygen of the air until the stain is fully developed — re-oxidizing any leuco-methylene blue and satisfying the reducing reaction of the tissue; then (4) either examining at once or fixing the stain *in situ* by its precipitation in an insoluble form for its preservation (imbedding and sectioning).

(1) The methylene blue may be brought in contact with the neurones by *injection*, (*a*) through the vascular system — aorta if the animal is small, artery supplying the part, if large; (*b*) into the body cavities; (*c*) subcutaneously; or, by *immersion* of the organ or part, or the entire animal if small (many invertebrates). If one keeps in mind the end result desired, the best method will suggest itself in a particular case. Cajal cut parallel slits in the cerebral cortex and inserted the methylene blue in powdered form or as a saturated solution. In general, the solutions should be as dilute as possible; of $\frac{1}{15}$ to $\frac{1}{4}$ per cent strength in physiological salt solution, the more direct the application the weaker. It is well to have on hand a 1 per cent stock solution in physiological salt solution and dilute it (with salt solution) as desired. A combination of injection and immersion is often advisable. In introducing the stain by injection, first remove the blood by washing out with physiological salt solution or by bleeding, and let the injection be full, i.e., through the capillaries into the veins. If a mammal is being dealt with, salt solution and staining solution should be warmed to body temperature (35–38° C.).

(2) It is difficult to give any general rules as to the time the methylene blue solution should remain in contact with the tissue before exposure, as it is best to determine it experimentally in each case. The time should be shorter for warm-blooded, longer for cold-blooded, animals. If introduced by injection, 20–30 minutes for a mammal, 2–12 hours for a cold-blooded form, may be suggested; the organ or part should then be removed, wet with the dilute stain (perhaps $\frac{1}{15}$ per cent strength) for another period of time — $\frac{1}{2}$–1 hour, access of oxygen to the point desired being kept in mind. At intervals, free-hand sections should be examined under the microscope to determine the state of the reaction. If the tissue is mammalian, it should be kept protected from evaporation during this time, and warm as in an incubator.

If the stain was applied by immersion, a shorter time suffices; up to 15 minutes or so with a subsequent exposure to the air of $\frac{1}{2}$–1 hour, wet with the dilute solution.

Small aquatic animals may be immersed in very dilute solutions ($\frac{1}{100}$ to $\frac{1}{1000}$ per cent), the optimum strength and time of immersion being experimentally determined.

In some instances (particularly parasitic worms) the tissue holds the methylene blue in reduced form in spite of exposure to the air, and the color is only developed when placed in the fixer (below). In any event it is better to fix the stain earlier than later.

(3) Two methods are standard for preserving tissue stained *intra vitam* with methylene blue. The first of these (Dogiel's) is suitable only for such tissue as may be exposed for study by teasing; the second (Bethe's) may be used both for such preparations and for those which it is desired to imbed and section.

Dogiel's Method. Immerse the tissue in a saturated solution of ammonium picrate (orange-yellow needles) for 2–24 hours according to the size of the piece, using abundance of fluid. If maceration occurs, Dogiel suggests addition of 1 per cent of 1 per cent osmic acid. Transfer tissue to equal parts of the ammonium picrate solution and glycerin, in which the tissue may be preserved, teased, and mounted.

Bethe's Method. Immerse the tissue in a 5–10 per cent aqueous solution of ammonium molybdate for 1–24 hours, according to the size of the piece, using abundance of the fluid. Trim the tissue as desired, removing all unnecessary parts, dividing it into smaller pieces, etc. Wash in distilled water, changed several times, for 1–3 hours. Dehydrate rapidly in 95 per cent and absolute alcohol — 4–6 hours, shortening the time if possible. Imbed rapidly in celloidin (§ 220) which may be hardened in 67 per cent alcohol and sections cut.

It may be advisable, particularly in summer work, to have the water and alcohol specially cooled to prevent dissolving of the stain in the alcohol. Tissue already fixed in the ammonium picrate may be refixed in the ammonium molybdate solution. Indeed, this double fixation is recommended by Bethe as particularly suitable for invertebrate material.

The paraffin method is not advisable if the above method suffices. If it is desired, however, after the dehydration, clear thoroughly with oil of cloves followed by xylene, and infiltrate in paraffin (§ 51). In treating the sections, avoid alcohol as much as possible.

Section or *in toto* staining may be applied, preferably carmine (not alkaline or acid formulas).

This important method has been elaborated largely through the work of Bethe, Cajal, Dogiel, Huber, Retzius and others, for which consult [10].

The method may be used for the staining of neurofibrillæ; for its applications for this purpose, consult the special articles.

224. Neuroglia Stain. Tissue is fixed for 24 hours in copper dichromate-sublimate-acetic ($\frac{1}{3}$ per cent) mixture (§ 16) and subsequently mordanted 3 or 4 days in 2.5 per cent copper dichromate. Imbed in paraffin. Sections (5–10 microns) are fastened to the slide and the Benda stain is used, as follows: (1) 4 per cent ferric alum for 24 hours; rinse well in distilled water and (2) place for 24 hours in a dilute solution (amber yellow) of sodium sulphalizarinate (conc. solution in 70 per cent alcohol added to distilled water). Rinse in distilled water, blot with absorbent paper, and stain (3) in a $\frac{1}{10}$ per cent aqueous solution of toluidine blue, heating it until it steams, cooling and staining 15 minutes. (4) Rinse with distilled water and treat for a few seconds with acid alcohol (70 per cent alcohol, 100 cc.; conc. HCl, 6 drops). (5) Blot with absorbent paper and dehydrate rapidly with 95 per cent and absolute alcohol. (6) Differentiate carefully with creosote, to the right degree; (7) blot with absorbent paper, and rinse in several changes of xylene. Mount in balsam. Neuroglia fibers, a dark blue, neuroglia "cells" a light blue, axis cylinder and myelinic sheath red to brown, nuclei dark blue, etc.

This method may be used with fresh or formalin material, human or animal. (Kingery.)

THE BLOOD

Special methods in the examination of the blood include (1) examining fresh blood; (2) technique of staining blood films; (3) determination of the number of red and white corpuscles per cubic millimeter; (4) differential counting of the white corpuscles; (5) determination of the relative amount of hemoglobin; (6) spectroscopic examination of blood (hemoglobin), etc. (1) and (2) are briefly given here; for (6) see [20].

225. Examining Fresh Blood. This consists in covering a drop on a slide, and immediately sealing the cover glass to prevent evaporation, observing the following cautions: (1) The drop of blood (from the finger or the lobe of the ear) should flow freely

and not be obtained by pressure. The drop should be a medium-sized one, which will spread out in an even, thin layer under the cover. (2) The drop should be received upon a cover or slide, covered, and sealed at once with castor oil.

Examination of fresh blood may be used in clinical examination for the detection of some abnormal conditions, and it is of value in the rough diagnosis of many others.

226. Stained Preparation of Blood. (*a*) *Preparing the blood film.* This may be best done in one of two ways: (1) The edge of a slide is first drawn through a drop of fresh blood and then moved quickly across the surface of a clean cover glass or slide, in this way spreading the blood in a thin, even layer upon the glass. With thin bloods (lower vertebrates, invertebrates), care must be taken not to crush. A non-absorbent paper may sometimes be used with advantage instead of a slide for spreading. Success depends upon getting the right amount of blood upon the edge of the slide, and the quick, even movement by which it is spread upon the cover glass or slide. Preparing the film on a slide is simpler and to be preferred if a differential count of the leucocytes is to be made. A second, possibly better, method is the following:

(2) Have ready two thin clean cover glasses (or slides) and obtain a drop of fresh blood. Take one of the covers in the forceps, touch it to the drop of blood, and place it upon the second cover glass eccentrically, with one edge projecting slightly. Slip the two covers apart in the plane of their surfaces and dry them quickly by waving them in the air or by passing them rapidly over the tip of a flame. The lower cover glass will have the better film.

(*b*) *Fixing the blood film.* If aqueous staining solutions are to be used, it is first necessary to fix the film; if, as is frequently the case, the stain is dissolved in methyl alcohol, no preliminary fixation is necessary. Blood films may be fixed while still moist or after drying, either by immersion in the fixing fluid, or by vapor. Orth's fluid (§ 17), Zenker-formol (§ 13), or methyl alcohol (strong) may be used, being applied directly. For vapor fixation, osmic acid, formalin, or a mixture of equal parts of tincture of iodin, formalin and 2 per cent osmic acid (Pappenheim) may be employed. If the last method is used, fix until the film is a light yellow. Rinse with 95 per cent and 50 per cent alcohols to remove the iodin and stain at once. A number of other fixers may, of course, be used. The application of heat (120° C. for 1–2 minutes)

for the fixing of dry films is indicated if Ehrlich's triacid mixture is to be used.

227. Staining the Films. Fixed films may be stained with hematoxylin and eosin as well as with other stains. If the film is on the slide, balsam and a cover glass are unnecessary if it is to be examined with the oil-immersion objective.

228. Eosin-methylene Blue. Most of the formulas are made on the principle of neutral stains, eosinates of methylene blue dissolved in methyl alcohol, the staining solution being diluted with water during the staining (§ 130). As polychrome methylene blue is generally used, the range of selectivity is increased by the presence of methylene azure.

For the staining of blood films with such mixtures as Wright's, Hasting's, and Jenner's stains, see §§ 129–131.

229. Blood of Arthropods. Wright's, Mallory's, Hasting's, and Giemsa's stains may be used, as with the blood of vertebrates, the film being spread in the usual manner. The time of staining must be determined experimentally, depending upon the kind of blood. A strong solution of iodin in potassium iodid, diluted with normal salt solution to the color of sherry, may be used for fixing leucocytes. A small drop of blood is covered and the fluid is allowed to run under the glass. Duboscq used a solution made up of 1 gram each of acetic acid, copper acetate, copper chlorid, osmic acid, thionin, and 400 cc. of distilled water. Some of this mixed with the blood of Chilopods fixes and stains in about 2 minutes. For direct staining, a drop of blood may be placed on a slide, covered with a cover glass, and allowed to remain $\frac{1}{4}$–$\frac{1}{2}$ hour; then a drop of a solution of $\frac{1}{2}$ per cent of methylene blue in a 1 per cent salt solution is run under the glass.

230. It is sometimes advantageous, as for class work and with non-mammalian blood, to handle blood in bulk. The following method has been used with good results. Fix for 1–6 hours by having the blood drop into a vial of 1 per cent osmic acid. The blood cells are allowed to settle and the supernatant fluid removed with a pipette. By this method the blood is passed through, successively, 2 or 3 changes of distilled water, 50 per cent, 67 per cent alcohols, paracarmine, 67 per cent, 82 per cent, 95 per cent and absolute alcohols, xylene, to thin xylene balsam in which the blood is stored. By gently agitating, the corpuscles are evenly distributed and a drop of the balsam, mounted, contains numerous

blood cells. This is an excellent method for preparing isolated epithelial and muscle cells for class use. Such material may be kept for years and is always ready to use.

231. Supravital Staining of Blood. Carefully cleaned slides (§ 170) are wiped out of alcohol and flamed. They are then flooded with 20–30 drops of a saturated absolute alcoholic solution of neutral red in 10 cc. of absolute alcohol, which is drained back into its bottle. The slides are dried in the air, and may be kept indefinitely. For mitochondrial staining, 3 drops of a saturated (absolute) alcoholic solution of Janus green is added to each cubic centimeter of the neutral red staining solution.

A film (not too thick) of fresh blood is prepared on such slides, sealed with salvolin or vaseline and paraffin, and examined on a warm stage or in a warm box with the maintenance of body temperature. For the differentiation of the various forms of leucocytes, which react differently to the dye or dyes, see F. R. Sabin: J. H. U. Hosp. Bull. 34, 277, 1923; Carnegie Inst. Embryol., 16, 165, 1925.

FINE INJECTION

For the purpose of examining microscopically the finer arteries and veins and the capillaries in a tissue, and their relation to the other parts, it is necessary to fill them with some colored injection mass, or otherwise stain or color them. Numerous injection masses are in use; the following meet the general needs. The first two formulas are suitable for use with warm-blooded animals. For the injection of cold-blooded animals, "cold masses" or solutions should be employed.

232. Carmine-gelatin Mass. *Formula:* Dry gelatin, 75 grams; carmine (No. 40), 10 grams; water, 90 cc.; ammonia, 10 cc.; acetic acid, *q.s.*; chloral hydrate, 10 grams.

Soak the gelatin in water until it is soft; pour off the superfluous water and melt it (in an agate or porcelain dish) over a water bath. Grind the carmine to a paste with water; add all the ammonia and water; filter, warm to 80 or 90° C., and add to the warm gelatin. Then add slowly the acetic acid diluted with an equal volume of water, while constantly stirring the mass, until the mass smells very slightly of the acid. Filter through fine flannel. If the mass is acid, the chloral hydrate may be safely added (as a preservative); if any ammonia is present it will decompose it,

forming chloroform and a granular precipitate. If too much acid is added, the gelatin will not set.

233. Berlin-blue Injection Mass. *Formula:* Dry gelatin, 75 grams; saturated aqueous solution of Berlin blue, 150 cc.; chloral hydrate, 10 grams. Prepare the gelatin in the manner given above (§ 232); warm the Berlin-blue solution (to 80° or 90° C.), and add it to the hot gelatin. Heat the mixture for 10 minutes or more, stirring it occasionally, filter it through fine flannel, and add the chloral hydrate.

234. For securing the best results in injecting, the following conditions should be observed: (1) A young but nearly mature, lean animal is to be preferred. (2) Kill the animal with an anesthetic (chloroform) and leave it in the anesthetic at least ½ hour before beginning the injection; do not, however, wait until *rigor mortis* sets in. (3) Inject only the part desired, tying all anastomosing vessels and all vessels to other parts. Inject into the artery of the part, leaving the vein open until nearly pure injection mass escapes, then tie it and continue the injection until the part feels hard and is the color of the injection mass. (4) When the injection is finished, cool the part injected by means of cold water, ice, or snow.

Harden the injected tissue 1 or 2 days in 50 per cent alcohol, 2 or 3 days in 67 and 82 per cent alcohols. The acidity of the alcohols should be insured by adding to the 50 per cent alcohol a few drops of acetic acid. The tissue may be stored in 82 per cent alcohol until ready for sectioning. Formalin (10 per cent) may also be used as the fixer and preserving fluid. For sectioning, the celloidin method is usually preferable.

235. Dense masses, such as do not pass through the capillary network, are useful in giving double injections, the veins and capillaries one color, arteries another. Two such may be mentioned: (*a*) lampblack gelatin mass; (*b*) ultramarine gelatin mass (Spaltcholz.).

(*a*) Lampblack and gelatin mass in the proportion of about 1 : 12.

(*b*) A 10 per cent gelatin mass to which is added ultramarine in proportion of 30 : 100.

In injecting, inject through the artery with one of the gelatin masses given in §§ 232–, and follow it up with one of the above, which will push the first mass through into the capillaries and veins.

236. Cold Gelatine Masses. By using only 5 per cent of the gelatin in the Berlin-blue gelatin mass (§ 233) an injection medium that is fluid at ordinary temperatures is prepared by adding slowly 5 or more grams of potassium iodid. Preserve the injected preparations in 5 per cent formalin.

Prussian blue in aqueous solution, alone or combined with gelatin (5 per cent) or glycerin (25 per cent), may be employed.

India ink (liquid) may be injected and has been used effectively in the injection or auto-injection of embryos.

Silvering blood vessels. Silver nitrate may be used for coloring blood vessels and thus differentiating them. See § 238.

The value of injection with masses that are opaque to the Roentgen ray and the production of roentgengrams for bringing out the larger blood vessels need only be mentioned in passing.

SILVER NITRATE IMPREGNATIONS

237. The preparations stained by means of nitrate of silver are made as follows: The *fresh* tissue is washed for a minute or so in distilled water to remove from the surface all albuminous substance, and then transferred for 2–5 minutes or longer to a 1–½ per cent aqueous solution of silver nitrate and exposed to direct sunlight until a light brown. When, by examination with the microscope, the stain is found to be sufficient, it is again rinsed in water and placed in glycerin or alcohol. Employed in this manner with fresh tissue, silver nitrate stains the cell cement, thus affording negative images of the cells. If a membrane such as mesentery is to be silvered after being removed from the body, it should first be cautiously stretched, as over a ring, to avoid creases.

238. Silvering Vascular Epithelium. In order that the vascular epithelium of small arteries, veins, and capillaries may be well demonstrated, silver nitrate solutions of ¼–½ per cent strength must be injected into the vessels.

239. Procedure. Connect a canula with the artery supplying the alimentary canal (superior mesenteric) or the brain (carotid) and inject distilled water until the water flows out of the returning vein colorless. Then immediately inject the silver solution until it runs from the vein. After a minute or two, follow with distilled water or physiological salt solution. Place the intestines and mesentery in water and expose them to the light until they become

slightly browned. Strips of the muscular coat of the intestines, especially of the rabbit, will show capillaries well. Veins and arteries side by side may be found in the mesentery. If the brain vessels are injected, one can get admirable preparations showing nuclei as well as cell outline, by staining in hematoxylin. Mount in glycerin, or, if desired, dehydrate and mount in balsam. The tissue may be kept in 50 per cent alcohol or in 50 per cent glycerin for several months before mounting if it is kept in the dark.

For large vessels and endocardial epithelium, open the vessels or the heart, and silver as directed above for mesentery. It may be necessary to make thin free-hand sections so that the preparation may be thin enough for high powers.

HISTO-CHEMICAL METHODS

There are special chemical substances which it is often desirable to preserve and differentially stain. In most cases, the staining reactions are not specific enough to come under the category of microchemical tests, the evidence gained being circumstantial or indirect, and the application of two or three different methods being sometimes necessary for confirmation. Such methods may be spoken of as histo-chemical rather than micro-chemical.

Fats (Lipoids)

240. Free fats and lipoids are soluble in ether, chloroform, absolute alcohol, xylene, benzene, and essential oils. As these are necessary for paraffin and celloidin imbedding methods, especially the former, the satisfactory preservation of these substances presents some difficulties. The use of the freezing microtome is therefore particularly called for. See, however, §§ 243, 244.

The stains applicable to the demonstration of fats are (*a*) stains soluble in the fat solvents, e.g., Sudan III and scarlet red; and (*b*) such as depend upon the reduction of salts by the fats (e.g., osmic acid and potassium dichromate). (See Faure-Fremiet, Mayer and Schaeffer, Arch. d'Anat. Micr. Vol. 12, 1910, pp. 19–102.)

241. Sudan III. Fix tissue in formalin or Müller's fluid. Cut free-hand sections, employ the freezing microtome method, or isolation. Rinse sections in 82 per cent alcohol and transfer

them to a strong solution of the stain in 82 per cent alcohol; leave several minutes covered from evaporation; rinse with 82 per cent alcohol and transfer to water. Mount in glycerin or glycerin jelly (§§ 155, 158). Fat is stained red.

242. Herxheimer's Stain. Preparation for staining is as above (§ 241). Pass sections into 67 per cent alcohol. Transfer to a strong solution of scarlet red in 67 per cent alcohol rendered alkaline by 2 per cent of sodium hydroxid. Stain for several minutes; rinse with 67 per cent alcohol and transfer to water. Mount in glycerin or glycerin jelly. Fat globules stained red. It affords a more intense stain than Sudan III.

Indophenol (saturated solution in 67 or 82 per cent alcohol) may be used as a blue stain for fat in a similar manner.

243. Osmic Acid. (§§ 31, 34). Osmic acid is reduced by the unsaturated fatty acids (e.g., oleic acid) which are blackened by it. The saturated fats and fatty acids (e.g., stearic, palmitic) are not so blackened but the black color subsequently appears when the tissue is placed in alcohol [48]. The fat so oxidized and impregnated with osmium (?) becomes less soluble in fat solvents (§ 240) and may be retained in tissue imbedded in either paraffin or celloidin. There is, however, a difference in fats; adipose tissue is easily preserved, while some of the fat granules found in the organs require the special precautions mentioned below.

Fix sections of tissue 2–3 mm. thick in Flemming's or Benda's fluid for 2 days; dehydrate in 95 per cent alcohol, and transfer to thin celloidin, and subsequently to thick celloidin, made up with 95 per cent alcohol (not absolute). The sections may be stained in safranin, quickly dehydrated and cleared with carbol-xylene, and mounted in balsam, either without a cover glass, or in thick balsam melted by heat and applied warm. Unless such precautions are taken, the solvent of the balsam may in time dissolve out the granules of blackened fat.

Paraffin does not afford as good a preservation of the more labile fat globules. Dehydrate in equal parts of 95 per cent and absolute alcohol, clear before the infiltration in carbol-xylene or chloroform. Paraffin in the sections should be dissolved out by carbol-xylene in preference to xylene (§ 145). In some instances, butanol (butyric alcohol) as a clearer before and after the paraffin, and the use of euparal (§ 162) as a mounting medium may be indicated.

244. Dichromate mordantage, with subsequent copper or iron hematoxylin stain, appears to rest upon the power of the fat or lipoid to reduce the dichromate and thus take on a mordantage which gives the basis for the subsequent staining. So far, it has not been possible by this technique to preserve, in paraffin or celloidin sections, the individual fat granules, but it is nevertheless a useful method for the differentiation of lipoid-containing cells.

Fix tissue 2 days in Zenker's with but $\frac{1}{10}$ or $\frac{1}{5}$ per cent of acetic acid or Helly's fluid, mordant 4 days or longer in Müller's fluid at 35–38° C., wash in water, imbed in paraffin, using chloroform as the clearer (§ 51). Stain sections with the copper hematoxylin (§ 97). Lipoid-containing cells, myelinic nerve fibers, erythrocytes, etc., are stained a dark blue. It should be remembered that other structures may also be stained by this technique.

The freezing microtome may be used with such tissue and stain, as has been done by Benda and Fischler [10].

Glycogen

Glycogen is soluble in aqueous media, and while it may be retained by a short fixation in several fluids, the best preservative of it is 95 per cent alcohol (82 per cent — absolute).

245. The Iodin Method. [19.] Fix tissue in 95 per cent or 82 per cent alcohol. Imbed in paraffin. Spread the sections, using, instead of the water, the iodin stain for glycogen, which is made up as follows: iodin, $1\frac{1}{2}$ grams; potassium iodid, 3 grams; sodium chlorid, $1\frac{1}{2}$ grams; distilled water, 300 cc. Spread sections may be stained or restained by immersing in the iodin solution, which will color the glycogen a mahogany red. For very soluble glycogen, 50 per cent alcohol may be employed instead of the water in making up the stain. In mounting, dissolve the paraffin with xylene, drain, place on the preparation melted yellow vaseline, cover, seal with shellac or balsam.

246. Best's Method. [4.] While rather complicated, this is generally recognized as the best method for the demonstration of small quantities of glycogen, especially when it is desired to see the relation of the granules to the protoplasm.

Fix tissue in 95 per cent alcohol; imbed (preferably) in celloidin (§ 63); if paraffin is used, after dehydration (§ 50) place the pieces of tissue in pure acetone for 15 minutes, xylene 20 minutes, xylene

paraffin 1 hour, pure paraffin (§ 51) 1 hour. Stain sections in Delafield's hematoxylin (§ 93) strongly, rinse and differentiate (if necessary) and stain in the following special carmine stain which should have been previously prepared. (*a*) Carmine, 1 gram; ammonium chlorid, 2 grams; lithium carbonate, 0.5 gram; distilled water, 50 cc. Bring the mixture to a boil. When it is cool, add 20 cc. of 10 per cent ammonia. Preserve the solution in the dark; after 2–3 days it is ready for use and retains its staining quality for a few weeks only. (*b*) When ready to stain, filter the above solution (*a*), and add to 2 parts of the stain, 3 parts of 10 per cent ammonia solution, and 6 parts of methyl alcohol.

Stain sections 1 hour, differentiate in a mixture of 2 parts of methyl alcohol, 4 parts of absolute alcohol, 5 parts of water; rinse with 82 per cent alcohol, dehydrate, clear, and mount in balsam. Glycogen stained an intense red.

In working with glycogen it is sometimes necessary to apply as a control the digestion of the glycogen in one or more sections or a part thereof, by means of saliva.

Amyloid

Amyloid, a form of connective-tissue degeneration, resembles glycogen in some of its physical (not chemical) properties and staining reactions. Two methods for its demonstration may be mentioned:

247. Iodin Method. Practically any fixer may be used (Zenker's fluid). Paraffin, celloidin, or (better) frozen sections may be used. Stain sections with the iodin solution (§ 245) for several minutes; rinse in distilled water and transfer to glycerin or glycerin jelly (§§ 155, 158) in which they may be mounted. Seal the preparations (§§ 165–). The amyloid a reddish-brown; the stain, however, will fade in the course of a few months.

248. Gentian Violet, among other anilin stains, colors amyloid differentially (metachromasia). Stain paraffin sections for several minutes in a 1 per cent solution of the stain; rinse and differentiate in 1 per cent acetic acid; wash thoroughly with distilled water, and (*a*) mount in glycerin jelly, or (*b*) dry in the air, treat with xylene, and mount in balsam. In the latter procedure, the staining may be applied before removing the paraffin.

Mucus

Mucous substances (mucins, mucinoids) possess acid properties and combine with alkalies and bases (heavy metals). Such combinations swell up or dissolve in water. Acetic acid, alcohol, and picric acid also precipitate these substances. For the fixation of mucus, most fixers may be used, Mercuric chlorid, Zenker's fluid, Flemming's fluid or Hermann's fluid. If, however, it is desired to preserve the mucus granules (mucinogen?) in the cell body, alcoholic or picric acid fixers are usually required, and the tissue at no time subsequently should be placed in water or aqueous solutions. Because of its acid character, mucus stains with basic stains, the first two methods given below being very selective. If cell granules are desired, remember the caution as to the avoidance of water.

249. Mucicarmine. (§ 106.) Fix as recommended above. Stain paraffin sections 1–24 hours; wash in water, dehydrate, clear, and mount in balsam. It may be used diluted with 50 per cent or 67 per cent alcohol.

250. Muchematein. (§ 94.) Employ in the same manner as mucicarmine. The alcoholic formula is indicated if cell granules are to be preserved.

Staining of the nuclei with hematoxylin (§§ 90, 91) or carmine (§ 101) or elastin stain (§ 126) may be previously given; a picro-fuchsin stain may follow it. Excess of alum (aluminium salts) or presence of acid must be avoided, hence wash thoroughly with water if there has been previous staining.

251. Basic anilin stains usually give sharp, often differential stains (metachromasia) for mucus. Methylene blue, toluidin blue, or gentian violet may be recommended. Safranin after Flemming's fluid fixation often gives a delicate stain for mucus.

Micro-chemical Tests

Micro-chemical tests for inorganic elements must occasionally be employed, particularly in cytological work. The methods have been largely elaborated by Macallum, to whose papers references are given.

252. Iron. (*a*) *Prussian blue reaction.* Alcohol fixation and the freezing microtome should be employed. Rinse sections in

distilled water, 2 per cent potassium ferrocyanate, 4–5 hours, 1 per cent HCl alcohol several hours, rinse in water, dehydrate, clear with clove oil; balsam. A carmine stain may be given, before or afterwards.

(*b*) *Ammonium sulphide reaction.* Place sections in ammonium sulphide solution (freshly prepared) for 5–20 minutes (dark-green color); rinse quickly with water, dehydrate, clear in clove oil, mount in balsam. It should be appreciated that other metals (silver, mercury, lead) will also give this reaction.

Free iron may also be demonstrated by the use of a pure aqueous solution of hematoxylin (dark-blue color); other metals, however, also unite with hematoxylin (e.g., calcium).

253. Masked iron does not give the reaction with pure aqueous hematoxylin [31, 34.] Macallum applies the ammonium sulphide at higher temperature (60° C.) and for several days.

254. Phosphorus. See Macallum's methods [34].

255. Potassium. See Macallum's method [36].

256. Chlorids. See Macallum's method [35]. Cautions!

257. Calcium stains strongly with aqueous hematoxylin and may often be so demonstrated. To identify the calcium and distinguish from iron, usual chemical tests may be applied [34].

Silver nitrate method. Tissue fixed in neutral formalin or alcohol may be imbedded, sectioned, and the sections treated with 1 per cent or $1\frac{1}{2}$ per cent silver nitrate solution for an hour or more. The presence of calcium is detected by black coloration. A nuclear stain (carmine or safranin) may be given if desired. Glycerin, glycerin jelly or balsam mounting may be employed. The test may be applied in the gross, and this is, of course, necessary if the amount of calcium is large.

257a. Incineration. The Policard method of obtaining by baking at high temperature a pure picture of the inorganic or salt content of the cells and tissues, is well presented by Scott in a number of papers: — Amer. Jour. Anat., vol. 53, No. 2, 1933, p. 243; Protoplasma, vol. 20, No. 1, 1933, p. 133.

SPECIAL METHODS FOR VARIOUS ANIMAL FORMS

In this section there are included only those methods which are applicable in specific cases for the preparation of the material for histological purposes, or for the making of whole mounts. Of the numerous methods which are in use, only a few of those which are of most general application can be given here. For other methods see Lee's Vade-mecum and Mayer's Zoömikrotechnik.

PROTOZOA

258. These are most satisfactorily studied while living. For retarding the movements, a 5 per cent solution of gelatin in water may be used. Use heat to dissolve the gelatin. A 1 per cent aqueous solution of chloretone may be used in some cases to narcotize the animals. For killing them, a 1 per cent aqueous solution of copper sulphate gives good results. A 1 per cent solution of formalin, a 5 per cent aqueous solution of acetic acid, a 10 per cent solution of corrosive sublimate, or a 25 per cent solution of tannic acid may also be used for this purpose. For *intra vitam* staining, make up saturated aqueous solutions of methylene blue, gentian violet, or safranin, and use aqueous dilutions of these to obtain the desired depth of color. For staining after killing or for killing with the stain itself, use aqueous dilutions of saturated alcoholic solutions of methylene blue, gentian violet, or safranin. A saturated alcoholic solution of iodin with 3 per cent of potassium iodid, when diluted with water, gives an excellent stain.

259. Marine Protozoa. To the water containing them, add a few drops of $\frac{1}{2}$ per cent solution of osmic acid, and allow them to settle. Stain with aqueous dilute picro-carmine. Many Radiolarians are best preserved with corrosive sublimate. Species of Sphærozoa may be fixed with equal parts of 70 per cent sea water with the addition of a little tincture of iodin or with a 5–15 per cent solution of corrosive sublimate in sea water. To the water containing delicate marine Infusoria, add a small quantity of a perchlorid of iron solution composed of one volume of 10 per cent alcoholic solution of ferric chloride diluted with 5–10 volumes of

70 per cent alcohol. Stain for 10–24 hours in alcohol containing a trace of gallic acid.

260. Soil Amœbæ. Take earth from several localities and cover with tap water, keeping it at 25° C. for several days. The bacterial membrane which forms will contain, in most cases, soil amœbæ. Fix for a minute or two a bit of the membrane in a mixture of 2 parts strong aqueous solution of corrosive sublimate and 1 part of absolute alcohol. Wash in iodinized alcohol. Or fix for 1 or 2 minutes in a saturated aqueous solution of picric acid, then wash in alcohol.

261. Protozoa in General. The film on the cover glass with the organisms is dropped face downwards into a fluid composed of 65 parts of saturated aqueous solution of corrosive sublimate, 33 parts of 95 per cent alcohol, and 2–5 parts of acetic acid, and left for 10–20 minutes. Wash for a few minutes in 50 per cent alcohol, then into 70 per cent alcohol (iodinized), then into 95 per cent alcohol for several minutes to several hours, after which return through the grades 70 per cent and 50 per cent alcohol to distilled water. Mordant for an hour or more in iron alum, wash, stain in iron hematoxylin for several hours, differentiate, dehydrate, clear in xylol, and mount in balsam.

SPONGES

262. Small Marine Sponges. Place the animals for 5 minutes in a 1 per cent solution of osmic acid and then transfer to strong alcohol, which should be changed twice. Sections may be stained in Mayer's alcoholic cochineal. Young Sycones may be fixed in absolute alcohol and stained with alcoholic carmine. Larvæ of Spongilla are allowed to settle on a large cover glass and then fixed for 3 minutes in absolute alcohol, stained in alcoholic carmine, dehydrated, cleared in oil of bergamot, and mounted in balsam. (Duncan, 1917.)

For larger species, fixation in absolute alcohol is strongly recommended. A weak alcohol-sublimate mixture or a picro-sulphuric solution also gives good results. Calcareous sponges may be decalcified in acid alcohol before imbedding.

Treat siliceous sponges with cold saturated solution of sodium fluorid in 40 per cent alcohol slightly acidified with a few drops of hydrochloric acid, leaving them in the fluid for several hours,

or in some cases, days. Sections may be stained with iron hematoxylin and acid-fuchsin.

CŒLENTERATA

263. Hydra. Fix the extended Hydra by quickly adding to a limited quantity of water, containing the animal, 2–3 volumes of hot (not boiling) Bouin's fluid. Fix for 30 minutes in cold Bouin's fluid, wash in alcohol, and preserve in 70 per cent alcohol.

Clearing and mounting hydra with ovaries and spermaries. After rapid dehydration, clear in wintergreen oil instead of xylene. Mount in glycerin.

264. Small Medusæ and Hydroids. Put the smaller and sensitive medusæ in a Petri dish with just enough sea water for them to swim freely. When they are fully expanded, add 2 drops of a 1 per cent solution of hydrochlorid of cocaine and then stir gently with a glass rod. Repeat at 5-minute intervals until there is no contraction of the tentacles on their being touched. Add 10–20 cc. of 4 per cent formaldehyde (10 per cent of commercial formalin), continuing to stir for several minutes. Without delay, to avoid the softening action of the cocaine on the jelly, transfer with as little water as possible to 4 per cent formaldehyde solution, where they remain for ½ hour or longer, and then store in 10 per cent formaldehyde solution (25 per cent solution of commercial formalin). In some cases a weaker solution (10 per cent commercial formalin) would be preferable.

Many specimens may be successfully preserved without the use of an anesthetic, by the gradual addition of formalin to the sea water containing them, the medusæ being kept in motion by very gentle stirring. Use about 1 part of 4 per cent formaldehyde to 10 parts of sea water, adding it drop by drop. Then treat as indicated above.

Menthol may also be used as anesthetic, as described for sea anemones. Preserve in formalin. For histological work, kill and fix the anesthetized animal in Bouin's fluid, or in a saturated aqueous solution of corrosive sublimate, washing it out as usual after these fluids. Subsequent treatment as for other tissues. (Duncan, 1917.)

265. Hydractinia are anesthetized by adding, drop by drop, a solution of 10 per cent chloreton in absolute alcohol until the

power of contraction is lost. Preserve in 4 per cent formalin solution. For histological preparations, stain with iron hematoxylin. Cut sections 7–8 microns thick.

266. Perigonimus and Gemmaria. Anesthetize with chloral hydrate or cocaine, fix in warm or cold mixture of sublimate and acetic acid or in strong Flemming's fluid, or in a 1 per cent solution of osmic acid. To preserve the shape, kill in 10 per cent formalin and transfer to 2 or 3 per cent formalin. Dehydrate very gradually. Stain in iron hematoxylin and orange G.

267. Medusæ. Fix in weak Flemming or, for some species (Carmarina), in a 6 per cent solution of sublimate in sea water, or a sublimate-formalin-acetic solution (45 : 5 : 2 : 50 water). Some forms cannot be fixed in sublimate solution since they cannot tolerate the iodin treatment. Material fixed in Flemming must be washed with great care as running water may loosen the epithelium. After dehydration, pieces of tissue are transferred through the several grades of ether-alcohol-celloidin, thence into chloroform to harden, trimmed closely, and imbedded in paraffin at 56° C. Stain sections in iron hematoxylin and in Mallory's fuchsin-anilin-blue-orange.

268. Leptomedusæ. For systematic work, fix in formalin-chrom-acetic; for purely histologic work, fix in Flemming's or Hermann's fluid.

269. Metridium. Narcotize with magnesium sulphate, fix by addition of much diluted chrom-acetic, and imbed after long infiltration. Stain sections in hemalum and borax-carmine.

270. Alcyonium and Gorgonia may be narcotized with menthol for 12–24 hours as described for Anemones, plunged into hot corrosive sublimate, washed in 70 per cent alcohol, iodinized alcohol, and stored in 70–80 per cent alcohol.

271. Anemones. Place the specimen in a glass dish with sufficient clear sea water to cover it to a depth of an inch or more, and then drop in crystals of menthol, which slowly dissolve. Depending upon the amount of water and the size of the animal, it will be sufficiently narcotized in 12–24 hours. Then plunge into formalin or formalin-alcohol. Cocaine may also be used, as described for medusæ. After narcotizing, the animal may be killed and fixed in Bouin's fluid or in corrosive sublimate and then treated in the usual manner (Duncan, 1917).

VERMES

272. Nematodes. A killing and dehydrating fluid is made up of absolute alcohol, 20 cc.; chloroform, 15 cc.; glacial acetic acid, 5 cc.; phenol crystals to raise the volume by 10 cc. Store in a glass-stoppered bottle. As the fluid deteriorates, it should not be kept over 2 weeks. The material may be most easily handled in Syracuse watch glasses, the edges of which are covered with paraffin. Slowly add cedar oil, wintergreen oil, or chloroform, to clear, replacing by pure oil in which the specimen may remain 10–15 minutes more. Add paraffin shavings, set in a warm place for 2 hours, then transfer to pure paraffin of 58° C., and imbed. Stain as desired. For whole mounts proceed as above, but clear, preferably in wintergreen. In the slightly inclined watch glass with the worm in a small quantity of oil, put a large drop of pure unthinned paper-filtered Canada balsam, and allow it to flow slowly into the oil and diffuse slowly for 2 or 3 hours. If diffusion is gradual enough, the worms will not collapse. Larger worms may be punctured before clearing. For glycerin-jelly mounts the worm should be treated in glycerin instead of the phenol fluid.

FIG. 12. — Syracuse Watch Glasses

Worms (nematodes, cestodes, trematodes, as well as some arthropods) which may have become collapsed may in many cases be restored by soaking in 35–50 per cent alcohol to which an equal volume of lactic acid has been added, the acid later removed by rinsing in 70 per cent alcohol. (Hetherington, 1922.)

273. Parasitic Nematodes. If the worm is killed in hot water or alcohol, it straightens, but staining is rendered more difficult. It is essential that the transference from one fluid to another be made gradually, preferably by means of Cobb's apparatus or by means of a string siphon differentiator (Magath, Trans. Amer. Micr. Soc. 35 : 245; May, Ill. Biol. Monogr. 5 : No. 2, p. 13). The flow is so regulated that 6–12 hours is required for one change of fluid to take place. Transfer through the weaker alcohols until 70 per cent is reached, then stain with Delafield's or Boehmer's hematoxylin in 70 per cent alcohol to which is added a little acetic acid. Continue through a weak solution of sodium or potassium acetate in 75 per cent alcohol, thence into higher grades of alcohol,

absolute alcohol, two or three xylene-alcohol mixtures, and xylene; then imbed and section. For whole mounts, transfer from the xylene to synthetic oil of wintergreen; or mount in balsam by adding to the specimen (which is in a thin layer of oil) bits of dry balsam, day by day, until of the proper consistency (May, 1922).

274. Nematodes. (*Acroboles.*) Relax and kill by heating in a hollow-ground slide in a few drops of distilled water. Fix in a mixture of distilled water, formalin, 90 per cent alcohol, and acetic acid (40 : 6 : 20 : 1). Transfer to 30 per cent alcohol with 2 per cent of glycerin, and evaporate for 6–10 days. For differentiation of the tissues, transfer from the fixative into Cobb's differentiator, through alcohols, stain with acid carmine, de-stain, then into absolute alcohol, thence through turpentine into balsam (Thorne, 1924).

275. Nematodes. (*Oxyuris.*) For total preparations, kill with 70 per cent alcohol heated to 68° C. After several days, dehydrate, then transfer to cedar oil: alcohol (10 : 90). Place in the exsiccator at 20° C. After three days, when the alcohol has evaporated, renew the cedar oil. For sections, fix in Flemming's, Altmann's, Benda's, or osmic acid fluid. Dehydrate slowly, slit the specimen, transfer to a mixture of cedar oil and absolute alcohol (20 : 80), place on the oven, and after a few hours transfer to pure oil, thence into equal parts of oil and paraffin, and finally into paraffin for 24 hours. Stain in hemalum or Delafield's hematoxylin, counter stain with eosin or orange G. Or, after Altmann's fixation, stain with Hansen's iron hematoxylin.

276. Gordius. For vital staining, use 0.001 per cent solution of methylene blue. For sections or for total mounts, fix in Schuberg's chrom-acetic acid, or Hermann's fluid, or Zelinka's fluid (1 per cent chromic acid, 35 parts; distilled water, 45 parts; acetic acid, 1 part). For glycerin mounts, transfer directly into glycerin; but for sections, first wash in running water; dehydrate by the drop method, and likewise pass slowly into xylene and paraffin. Cut embryos 5 microns; larvæ, 3 microns. Stain in Ehrlich's hematoxylin, or in anilin-gentian-violet (5 cc. of a saturated solution in 95 per cent alcohol to 100 cc. anilin water) for 24 hours, then differentiate. Mount young larvæ after fixation in Hermann's or Zelinka's fluid without washing, by slow transfer, into glycerin.

277. Planarians. Allow planarians, which have been without food for several days, to extend on a glass slide; draw off most of the water, and then wholly cover the animal with 2–3 drops of 2 per cent nitric acid. After death, cover with Gilson's fluid or some other mixture containing corrosive sublimate, then transfer to the fixing fluid for 30–60 minutes (Hyman, 1924).

278. Trematodes. To kill trematodes, compress specimens between slides to keep the worm in a distended condition, then run hot alcoholic-corrosive-acetic, Zenker's, Bouin's, or lacto-phenol fluid between the slides. *In toto* mounts and sections may be stained with borax-carmine or Delafield's hematoxylin (Barker and Parsons, 1917).

279. Small Oligochætes. Whole mounts of Oligochætes and some nematodes may be made by placing them on a slide or in a watch glass and adding salt solution until the mixture reaches 1–3 per cent; then kill the animal on the slide under the cover glass over a flame, avoiding bubble formation through over-heating. Small specimens may be fixed in various fluids under the cover glass; those over 1 mm. in length, in the watch glass. Results are not so good for worms secreting much mucus.

280. Earthworms. Place the worms in a dish containing moist filter paper. After a few days the contents of the alimentary canal have been replaced by the paper. Coffee grounds or tea leaves may be used instead of the paper. Next transfer the worms to a shallow dish, covering them with water. Add a little alcohol from time to time, until at the end of an hour or two the strength of the liquid has been increased to about 10 per cent. When the worms have been completely narcotized, wash, and replace in 10 per cent alcohol.

Worms for dissection. From the 10 per cent alcohol, place the worms in a 1 per cent chromic acid solution, then inject the acid into the body cavity until the worm becomes turgid. The injection must be done slowly, the hands protected by a coating of vaseline. At the end of 4 hours, wash thoroughly in running water for 12 or more hours. Then transfer through 50 to 70 per cent alcohol, 2 or 3 days in each, preserving in 70 per cent.

Material to be used for sectioning is placed in Zenker's fluid, after the preliminary treatment in 10 per cent alcohol as indicated above. After 4 or more hours it is treated as usual after this

reagent. The worm should be slit in order that the fluid may more readily penetrate the tissues.

281. Earthworms. (Pharyngeal glands of Allolobophora and Lumbricus.) Fix in Bouin's fluid or Schaudinn's fluid with 3 per cent acetic acid, followed by staining in Mayer's hemalum or glychemalum with eosin or orange G as a counter stain, or in

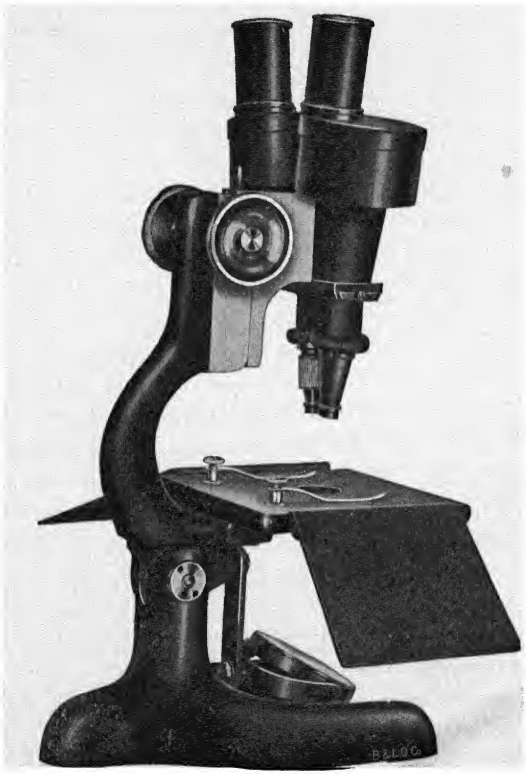


FIG. 13. — Bausch and Lomb Binocular Microscope KF for dissecting and taxonomic work

magenta-red and picro-indigo-carmine. For a study of the glandular secretion, use metachromatic stains, thionin and toluidin blue in accordance with slightly modified methods of Hoyer and Hári (Arch. mikr. Anat. 36 : 58); or a purely mucin stain, a 10 per cent solution of mucihematein (P. Mayer) for 2–5 minutes after fixation in Bouin's fluid, either with or without previously staining with

iron hematoxylin. For more delicate structures of the gland and pharyngeal epithelium, fix small pieces in Champy's chrom-osmic solution and stain with iron hematoxylin and eosin (Keilin, 1919).

For cytological study of the photoreceptors, earthworms are fixed in strong Flemming's solution and stained in iron hematoxylin. For a study of the general structure, fix for 24 or more hours in a solution of 20 per cent formalin containing 3 per cent glacial acetic acid. Impregnate 10–18 days in 2 per cent silver nitrate at 37.5° C. in the dark. Develop for 18–36 hours in 2 per cent hydroquinone, in the dark. Dehydrate from 70 per cent alcohol through 85, 95 per cent, and absolute, then clear in xylene and mount in balsam (Hess, 1925).

282. Rotifers. Narcotize a mass of rotifers in water in a watch glass with a solution of cocaine (hydrochlorate of cocaine, 2 per cent solution, 3 parts; methyl alcohol, 1 part; water, 6 parts). When movement ceases, draw off the water with a pipette and add a drop of a $\frac{1}{8}$–$\frac{1}{2}$ per cent solution of osmic acid, and after a minute wash in several changes of 2.5 per cent formalin, preserving in a 3–6 per cent formalin. If too much blackened with the osmic acid, the material may be bleached with peroxid of hydrogen. The specimens may be mounted on a hollow-ground slide in 2.5 per cent formalin. Ring the cell first with a thin ring of copal varnish, then several coats of a cement made of a mixture of gold size, shellac, and India rubber, finally finishing with three more coats of gold size, allowing 24 hours to elapse between each application. Instead, the first ring may be made of a mixture of $\frac{1}{3}$ gold size and $\frac{2}{3}$ gum damar, then two coats of pure shellac followed by three coats of gold size.

These animals may be advantageously studied alive as directed for Protozoa (§ 258).

283. Bryozoa. Anesthetize with cocaine as described for Medusæ, and then kill with 10 per cent solution of formaldehyde (25 per cent formalin), taking care to transfer the calcareous forms promptly to 70 per cent alcohol.

Preserving fresh-water Bryozoa. Place the colony in water in such a way that it may not come in contact with the sides of the container. Narcotize with a saturated solution of cocaine or chloreton dropped from a pipette and allowed to diffuse, adding more at intervals of 5 minutes. In half an hour the colony may be found fully expanded. When this stage is reached, about 5

per cent of the entire fluid may be withdrawn and replaced by cocaine solution forcibly injected. After the ciliary movement has ceased, no further cocaine need be added. After complete narcotization (perhaps 1½ hours) wait a little longer and then add preserving fluid, made up of 10 parts of cane sugar and 90 parts of distilled water, then adding 2 parts of formalin. The preserving fluid is to be added, a pipette full at a time, until there is about 50 per cent sugar solution in the mixture; then leave it for ½ hour. Add more fluid more rapidly, leaving it for ½ hour or longer, then transfer to undiluted fluid. After a week or ten days, replace with fresh fluid. For sections, take small pieces, preserved as above, and place in Bouin's fluid for several hours. Subsequent treatment as usual with this fluid (Jackson, 1917).

ECHINODERMATA

284. In general, these animals may be fixed in the usual fluids and preserved in 90 per cent alcohol. Decalcify, before sectioning, in 70 per cent alcohol which has been acidified with hydrochloric or nitric acid. Free the material from the CO_2 by allowing it to remain in alcohol for a day or more, using an air pump if necessary.

Larvæ of Auricularia, Bipinnaria, and Pluteus are best killed in cold saturated aqueous solution of corrosive sublimate and fixed for not exceeding 4 minutes in the same fluid. Stain for 12–24 hours in Mayer's old alcohol cochineal stain, diluted with 70 per cent alcohol until only a trace of color remains; then dehydrate, clear in cedar or clove oil, and mount in balsam. Over-staining may be reduced (if the specimen has not a delicate calcareous skeleton) with acidulated alcohol or 1 per cent acetic acid. Narcotize Holothurians with menthol as described for Anemones, then transfer to 70 per cent alcohol and at the same time inject 90 per cent alcohol through the anus. Store in 70 per cent alcohol (Duncan, 1917).

MOLLUSCA

285. For obtaining specimens in an expanded condition, it may be necessary to anesthetize them. This may be accomplished with 70 per cent alcohol, or 2 per cent cocaine in water, or a 0.1–1 per cent solution of chloral hydrate. Or they may be placed in a covered vessel in tepid water which has been boiled (to drive off

the oxygen). The addition of chromic acid in quantity sufficient to tinge the water deeply may be helpful.

The alimentary canal of the snail (Helix). Fix in a mixture of Flemming and 7–14 per cent formalin. Stain with iron hematoxylin and counter-stain with acid fuchsin, or stain with Delafield's hematoxylin, or safranin.

Muscle tissue in snails. Anesthetize snails in water that has been boiled. They should be allowed to remain at a temperature of 37° C. From the anesthetized animal, cut off the head and part of the body and fix in Zenker's fluid. Imbed in celloidin and cut 6 microns thick and upwards. Stain in iron hematoxylin. Harden isolation preparations in $\frac{1}{2}$ per cent chromic acid and examine, either stained (hematoxylin) or unstained, in glycerin water.

286. Nervous System of the Snail. Dissect out a part of the central nervous system of a living snail and fix for 24–48 hours in strong Flemming's fluid. Stain in safranin and light green. After saturated sublimate fixation (without acetic acid) stain in Ehrlich's triacid or iron hematoxylin. A ganglion quickly removed from a 1-year-old animal may be treated with methylene blue solution (0.00001 gram in Locke's mixture) for 24 hours at room temperature, then 24 hours in ammonium molybdate, then washed, imbedded in paraffin, and cut 30–40 microns thick. Or, the freshly dissected-out ganglion may be fixed for 2 hours in 40 per cent formalin and 5 per cent copper sulphate (1 : 2); washed for 24 hours and imbedded. Stain for 10–15 minutes in Mallory's phospho-molybdic acid hematoxylin. Differentiate in 40 per cent alcohol, completing in 60 per cent ammonia alcohol.

287. Reproductive organs of the slug (Limax). Remove embryos from their envelopes, wash in saline solution, then fix in Zenker's or Hermann's fluid. Allow recently emerged young to extend in the dish, then pour Zenker's fluid over them. Allow older animals to extend in distilled water for 8–10 hours. Fix in sublimated nitric acid or other fluids. Imbed young embryos through chloroform, older ones through xylol into paraffin. Remove the reproductive organs from young animals, and imbed in clove-oil-celloidin. Cut sections 4 microns thick. If albuminoids in the head and liver prove too brittle, place the partly cut paraffin block in water to soften and then continue cutting. Stain in iron hematoxylin.

TUNICATA

288. Ascidians with contractile zoöids are allowed to expand in clear sea water, narcotized with menthol as described for Anemones, and then plunged into a solution of acetic acid, where they should be left for 3–10 minutes according to size, removed with wooden forceps or with the fingers, washed in 50 per cent alcohol and dehydrated in the usual way. Salpa, Pyrosoma, and other free-swimming Tunicates may be killed and preserved in 10 per cent formaldehyde. A small quantity of 1 per cent solution of chromic acid added to the final formalin solution will help to harden the gelatinous test (Duncan, 1917).

ARTHROPODA

289. When it is impossible or inexpedient to remove the chitinous integument of an arthropod, the object must be fixed in a fluid that penetrates rapidly. Of these, Gilson's, Petrunkevitch's, Carnoy's, Carnoy and Le Brun's, Bouin's, and Hennings' fluids are frequently indicated. In many cases they may be used hot (60–80° C.), thus securing fixation by heat as well as facilitating penetration. Whenever it is feasible, however, the body wall should be slit or pierced in places where no serious injury results to the parts to be studied. In this case, any of the usual fluids used for fixing tissues may be employed. For methods of dealing with chitin, see §§ 183–187.

290. Tracheal System. In dissections of fresh material, tracheæ are readily recognized by their glistening white appearance, due to the air which they contain. When immersed in water or preserving fluids, they fill up after a time, the smaller branches being then less easily distinguished from nerve fibers. For injecting the trachea, place the insects in a flask and pour in just enough India ink to cover them, in some cases first removing the wings. Connect up the flask with an aspirator which is attached to a common water faucet. On turning on the water, the air is withdrawn from the flask by the aspirator, creating a vacuum. At the same time the air is withdrawn from the tracheæ of the insects, and is replaced by the India ink. On opening the insect, the tracheæ stand out clear and black (Dusham, 1914).

291. The method for the injection of the tracheal system given by Krogh of Copenhagen is more delicate (Vidensk. Medd. fra

Dansk Naturh. Foren. 68 : 317). An injection mixture is made up as follows: Alkanna root digested with turpentine for at least one week, preferably at about 30° C. Filter the extract and add 50° C. paraffin and beeswax in equal parts with a little colophonium to diminish the brittleness. The mixture should melt at about 40° C., though one with a lower melting point penetrates more easily. For larvæ of cockchafers, colored turpentine is the only fluid that can be used. Connect up the apparatus as shown in the figure, the injection mixture in bottle (1) of 250-cc. capacity, with ground-glass tubulate stopper. The animal is deeply nar-

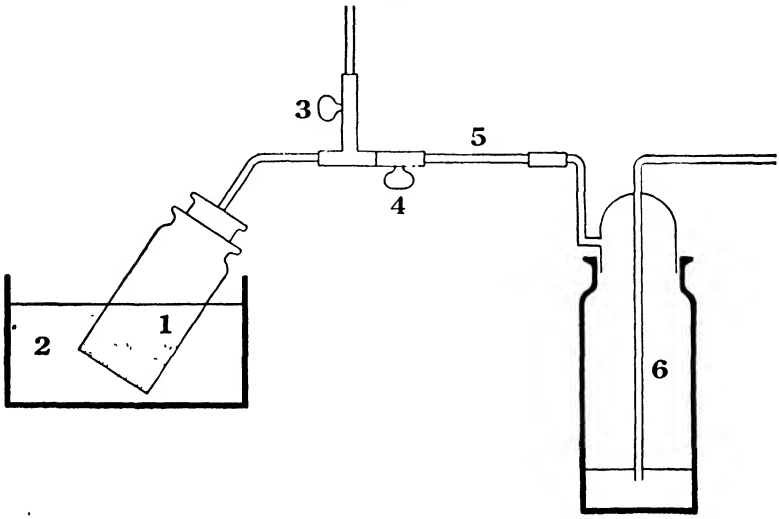


FIG. 14. — Krogh's Apparatus for Injection of Tracheæ

cotized (not killed) with ether. It may be necessary to introduce hairs into the spiracles to keep them open. Weigh the animal down with lead or copper wire and put it into bottle (1), grease the stopper well and then connect up with the air pump or aspirator. After a few minutes, place the bottle in the water bath (2) at a temperature of 40–50° C. The injection substance melts and the animal sinks to the bottom. When the substance is completely melted the valve (3) is closed and the pump disconnected. By means of the valve (4), a connection is established with the wash bottle (6) in which there is a little water. The thermometer tube (5) has a bore of not over 0.1 mm. When the valve (4) is opened slightly, air should bubble in *very* slowly

through the wash bottle and the animal will sink and become injected. Increase in pressure should be slow. After ½ hour, open the valve a little more; but the injection should not be complete in less than 1 hour. When the pressure is back to normal, fish out the animal and cool it quickly in water. Remove with ether any of the substance sticking to the insect.

Mount the insect on a slide with picene (picein). Heat the slide cautiously over a small flame until the slide is completely covered with a good layer of picene, into which the underside of the animal is stuck. When the picene has set by cooling, open either the dorsal or the ventral side, as desired, on the middle line, and spread out, sticking the body wall into the picene, which is gently heated for the purpose.

292. When the tracheal system alone is desired, other parts may be removed by digestion. Place the mounted preparation in water to which has been added about 5 per cent of hydrochloric acid (1 cc. of conc. HCl to 100 cc. of water) and ½–1 per cent of commercial pepsin. Set aside in a warm place (25–35° C.) and leave for 2–3 hours. Take out the preparation and wash gently under the tap. Repeat the process of digestion and washing, until the desired stage is reached. Preserve the preparation in weak formalin solution. The dye will not stand strong light.

293. The minuter branching of the tracheal system may be very clearly demonstrated on the surface of the dark-colored crop and proventriculus of Dytiscus larvæ by means of Köppen's technique (Zoöl. Anz. 52 : 1912). Living larvæ are kept alive for two weeks in a large closed vessel containing a small vial of a 2 per cent solution of osmic acid. Pieces of the wall of the proventriculus and of the fat body, etc., spread flat on a slide and mounted in glycerin jelly, will show the branching tracheoles in some cases. The action of the osmic acid seems to prevent the body fluids from filling the tracheoles, and thus the presence of the air renders them visible. Though the tissues are blackened by the reagent, the tracheoles do not seem to be especially affected.

294. The tracheoles may also be demonstrated by the use of the Golgi chrom-silver method in some cases. Or, after being fixed in alcohol, they may be treated with glycerin and washed, then placed in a ½ per cent solution of gold chlorid for ½–1 hour and reduced in a 1 per cent solution of formic acid for 10 days or more. These methods are capricious.

Nervous System. For a study of the nervous system, and especially of the brain structure, fixing fluids containing a considerable amount of alcohol are best avoided, as the stains will not sufficiently differentiate the fibers. The following methods bring out the finer structure of the brain, the mushroom bodies, ganglionic cells, etc.

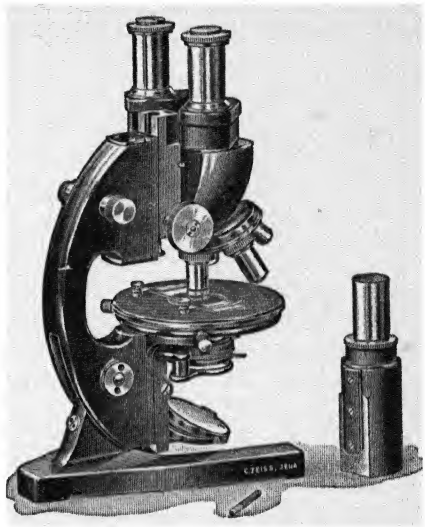


FIG. 15. — Zeiss Monobjective Binocular Microscope

295. Fix the brain of an insect of moderate size in 10 per cent formalin for 4–20 hours (in most cases 6 hours). After dehydration, clear in xylene and imbed in paraffin. Stain in eosin for 20–30 seconds; Delafield's hematoxylin, 30 seconds; wash in water and then transfer to phospho-molybdic acid (1 per cent), for 2–3 minutes; wash in water; Mallory's fluid, 6 seconds; wash in water; dehydrate, clear and mount. Or, after fixation in formalin, wash in water, then in 5 per cent copper sulphate for 24 hours; wash, dehydrate, and imbed. Stain the sections in a 10 per cent solution of phospho-molybdic acid, 1 cc.; hematoxylin crystals, 1 gram; chloral hydrate, 6–10 grams; water 100 cc.

296. For vital staining (§§ 87, 223) inject methylene blue, in varying concentrations up to $\frac{1}{2}$ per cent in $\frac{3}{4}$ per cent salt solution, by means of a small syringe, into living larvæ of moths or beetles. Insert the canula only under the hypodermis and muscles and not into the alimentary canal, injecting enough liquid to color all segments. After injection, the animal is left quiet for a period of 2–4 hours, during which the insect must remain alive. After successful staining, the specimen is cut open lengthwise and examined in salt solution for the course of the nerves, which are stained blue. The color fades after an hour or two, though in some cases it may be fixed for a limited time in a 10 per cent solution of ammonium molybdate.

297. The Egg and Embryo. In preparing the eggs of arthropods for sectioning, it has been the experience of most investigators that piercing the chorion and vitelline membrane is necessary to permit the penetration of the usual fixing fluids. In many cases the ova are best killed and fixed by dropping them into hot fluid (60–80° C.). When cool, pierce the egg chorion with a fine needle, continue the fixation for the necessary period and then preserve the object in 75–80 per cent alcohol. Before complete dehydration and imbedding the chorion should be removed if possible. To do this may require some experimentation owing to differences in thickness or toughness of eggs of different species.

298. In the earlier stages, the large amount of yolk presents the added difficulty of brittleness, especially in eggs that have been preserved for some time. Soaking them in water for 12 to 24 hours in some cases facilitates cutting, although it may be necessary to resort to imbedding in paraffin-celloidin to prevent the crumbling of the sections (§ 72). Paraffin-rubber-celloidin (Hance, Science, 77 : 353, 1933) in some cases has given favorable results. In place of crude rubber, Devoe and Reynolds rubber cement is a good substitute provided the stock solution is kept heated until the odor of the benzene is no longer given off. Refractory material has also successfully been handled by the use of Petrunkevitch's phenol fluid as described by Slifer and King (Science, 78 : 366, 1933). The use of *n*-butyl alcohol for dehydration has also given excellent results (Stiles, Stain Technology, 9 : 97, 1934). Spherical eggs may be stained lightly in Mayer's paracarmine or in borax carmine, and then, after clearing in cedar or clove-oil, the later-stage embryos may be seen through the chorion and will thus permit orientation. In many cases the embryo may be dissected out, oriented and imbedded.

299. Preserving Arthropods Entire. See Banks' Directions ('09).

300. Slide Mounts. To make a slide mount of an entire insect or other small arthropod, or of the appendages, fresh, dry, or alcoholic specimens may be used. If highly chitinized or deeply pigmented, the specimen should be soaked in 5–10 per cent caustic potash until the parts become sufficiently light-colored and transparent. If time is limited, it may be boiled for a few minutes in the potash in a casserole over a Bunsen burner. When clear and free from tissue, it should be soaked in water to remove the alkali. Dehydrate through an alcohol series of 30, 70, 95 per cent, and

absolute, leaving the specimen 10–15 minutes in each. One of the series may have a trace of hydrochloric acid. If it is necessary to stain the specimen, acid-fuchsin in 95 per cent alcohol, immediately before the 95 per cent alcohol of the dehydrating series, may be used. After dehydration, clear in xylene, then place the specimen on the middle of a slide and cover with a small drop of moderately thick balsam, setting it aside under a bell jar. After 24 hours add a little thin balsam and then put on the cover glass. If immediately covered after being placed on the slide, the specimen is liable to be displaced. Three bits of glass placed around the specimen before putting on the cover glass will keep the latter in a horizontal position. When dry, scrape off the excess balsam, then clean with a cloth moistened with xylene. The appearance of the mount is improved by striking a ring of gold size or Brunswick black around the cover glass, using a turntable (§ 154). A label giving all necessary information, such as name of object, locality of capture, etc., should be put on one end. For hints on labeling, see §§ 166, 167.

FIG. 16. — Turn Table

Freshly captured specimens that are not heavily chitinized or deeply pigmented may be put directly into 70–80 per cent alcohol and then dehydrated, cleared, and mounted.

301. Preserving Fluids. *Alcohol.* In general, 80–85 per cent grain alcohol is the most useful liquid for preserving soft-bodied arthropods, spiders, immature stages of insects, and the like, for taxonomic purposes. Well-chitinized forms may be dropped at once into 85 per cent alcohol, which kills and preserves at the same time. Soft-bodied larvæ of Coleoptera, Diptera, Hymenoptera, and hairless caterpillars may be killed in hot water and then placed in 50 per cent alcohol for a few hours, then into 70 per cent for an equal time, and finally in 85 per cent for keeping. The addition of 0.5 to 1 per cent glycerin is considered by some to be an improvement. It is certainly a protection to the specimen should the alcohol evaporate due to a defective cork. If the insect is large it is advisable to slit it on one side to permit penetration of the preserving fluid. Very delicate larvæ may, after killing in hot water, first be placed in 50 per cent alcohol, then into 70 per cent, and finally into 85 per cent for preservation. To preserve specimens that are later to be used for dissection, it is especially desirable to slit them on one side

to insure better preservation. As the body fluid of the insect dilutes the preservative, it is necessary to replace the alcohol by fresh fluid after a day or two, when the quantity of fluid is small relative to the size of the specimen. The objects may best be kept in homeopathic vials with sound corks. Very small ones may be put in short lengths of glass tubing or minute shell vials with cotton stoppers, and these stored in larger vials or jars. Specimens that have become dry may in many cases be restored by soaking in warm water before replacing in alcohol. Small thin-skinned larvæ that have become dry and flat may be caused to become plump again by a short stay in 5 per cent caustic potash, and then gradually dehydrated. Diaphanol may be used for the same purpose.

Formalin. A 10 per cent solution of commercial (40 per cent) formalin is much used as a preservative. It is also used in a 5 per cent solution. Bottles containing it should be tightly corked to prevent deterioration. The addition of ½ per cent glycerin renders the material in it more flexible.

Choral hydrate. A 5 per cent solution of chloral hydrate preserves the internal organs and also leaves them flexible. Specimens may be killed by dropping them into the fluid where they should remain 24 hours. They are then taken out, slit open at some point where it will cause least injury, and placed in fresh fluid for final preservation. This is an excellent means of keeping specimens that are to be used for a study of internal anatomy. The delay of one day before making the slit in the body is important in order to avoid excessive muscle contraction.

302. Dry Mounts. By far the greater number of insects to be used for taxonomic purposes are mounted on pins. It is not the aim here to describe in detail the various methods used in museums. For an account of these the reader is referred to Banks' Directions (Bull. 67, U. S. National Museum, 1909).

PLANKTON ORGANISMS

303. The plankton consists of Algæ, Protozoa, Rotifera, small Crustaceans, etc. In case the catch cannot be examined at once it is convenient to preserve it in some fluid. A number of formulas for this purpose are given below.

(1) *Formalin.* A 10 per cent solution of the commercial formalin. This preserves well the colors of the organisms. The bottle must be well corked to prevent deterioration.

(2) *Alcohol sublimate.* Distilled water, 100 cc.; mercuric chlorid, 3 grams; 70 per cent alcohol, 100 cc. Put the organisms into this for 4 hours, then wash in 70 per cent alcohol to which a little tincture of iodin has been added to give it a deep yellow color. Renew the fluid until the color of the iodin is no longer affected. Preserve in 80 per cent alcohol.

(3) *Flemming's strong solution* (§ 34). Put the plankton concentrate into this for 10 minutes or more. Wash in 70 per cent alcohol for ¼ hour. Preserve in 80 per cent alcohol.

Cladocera may be preserved in 95 per cent alcohol. Soft-bodied forms may be killed in chloral hydrate or osmic acid. To mount, put the specimen in a small drop of glycerin on the center of a slide, with three bits of glass just thick enough so that when the cover glass is put on it rests lightly upon

the object. When the cover is put on, the glycerin should only occupy the center. Place on a bit of paraffin, melting point 50° C., and warm the slide. The paraffin will run under the cover and thus seal the object. For a permanent mount, the cover should be cemented on with some microscopic cement.

For Copepoda, Marsh recommends 75 per cent alcohol as a preservative. Dissections should be made in glycerin, and the parts mounted in Farrant's solution. When completed, the cover should be sealed with Brunswick black or some similar cement.

Ostracoda may be narcotized in a 60–70 per cent alcohol and then transferred to 80–85 per cent. For study, the shell must be removed and the parts mounted as with the Copepods.

Water mites (Hydracarina) may be preserved in a fluid made of glycerin, 2 parts; water, 3 parts; acetic acid, 2 parts; absolute alcohol, 1 part.

Crustacea, marine Copepoda, Ostracoda, Amphipoda, Isopoda, Cirripedia, and larval stages of Crustacea generally may be treated with 5 per cent formaldehyde in sea water and then transferred to 70 per cent alcohol. Stain with weak alcoholic picro-carmine, clear with terpineol. Avoid over-staining. Mount directly in balsam or wash first in benzol and mount in balsam.

304. Staining and Mounting Plankton Organisms. Place several living Crustaceans (Cyclops, Daphnia, etc.) in 30 per cent alcohol for 5 minutes, then transfer to a watch glass containing a few drops of hemalum. After 5 minutes (more or less) transfer to water. The animals should appear deep blue. If not sufficiently colored they may be returned to the stain: if over-stained, they may be de-stained in acid alcohol. Remove the specimens from the stain and place in acidulated alcohol for a minute or two, until they assume a red color, then rinse in water for 5 minutes, then transfer to a $\frac{1}{2}$ per cent aqueous solution of bicarbonate of soda to restore the blue color. Wash again for 5 minutes in distilled water, then dehydrate through the grades of alcohol, 30, 50, 70, 85, and 95 per cent, and absolute, 10 minutes in each. Clear in clove oil, which will require but a few minutes. When clear, transfer to a clean slide into very thin balsam. Set the slide aside in a dust-free situation until the balsam has thickened a little, put on thick balsam, and cover. In order that the cover glass may not bear on the object, put in bits of glass the thickness of the object to support the cover. The appearance of the slide is enhanced by sealing with gold size when the balsam is sufficiently hard.

Certain small Crustaceans may also be stained in borax carmine until they assume a deep-red color. Wash in 70 per cent acid alcohol, thence into 80 per cent alcohol.

Living organisms that are difficult to see, owing to their transparency, may be rendered more distinct by putting into the water a trace of methylene blue, only sufficient to tinge it faintly. Some of their tissues take up the stain, but since they soon lose it again the creatures must be examined from time to time. A reddish-yellow solution of neutral red may also be used for vital staining. Place the vessel containing the organisms and the stain in a dark chamber for 2 hours and then examine. Vital stains are not permanent.

TAXONOMIC MATERIAL

305. Spiders and Myriapods. Most collectors place spiders directly in 90–95 per cent alcohol without further treatment. Some workers prefer to add about $\frac{1}{2}$ per cent of glycerin, maintaining that this renders the specimens more flexible and, in the event of a loose cork, prevents total evaporation of the fluid and consequent drying out of the specimens.

Minute Arthropods, such as mites collected in moss, earth, etc., with a Berlese trap or similar contrivance, may be preserved, according to Krause (Mikrokosmos 9 : 266, 1915) in Oudemans' mixture, composed of 87 parts of 70 per cent alcohol, 5 parts of glycerin, and 8 parts of acetic acid; then mounted for examination in a mixture of 50 parts of 70 per cent alcohol and 50 parts of glycerin.

For Orbatidæ, Willmann (loc. cit., 9 : 225, 1915) recommends 45 parts of clove oil, 35 parts of 95 per cent alcohol, and 20 parts of glacial acetic acid. It tends to stiffen the objects, but they may be transferred directly to Canada balsam.

For Protura, etc., Krause (Mikrocosmos 10 : 23, 1916) recommends fixation in absolute alcohol for 4 days; after which the specimens should be transferred to fresh absolute alcohol, then into a mixture of a one-third xylene and two-thirds absolute alcohol for one hour; then into a mixture of equal parts of alcohol and xylene, 3 hours, then pure xylene for $\frac{1}{2}$ hour. Mount in Canada balsam.

306. Collembola. Guthrie (1903) states that these insects may be collected directly into 80 per cent alcohol or into 80 per cent alcohol to which 10 per cent of glycerin has been added. Or they may be killed by pouring hot 95 per cent alcohol on them.

For a study of parts, they may be dissected and examined in glycerin. For a permanent mount, dehydrate through 95 per cent and absolute alcohol, clear in cedar oil, and mount in damar balsam. When necessary to de-pigment, place a specimen under a cover glass, run in a drop of 5 per cent caustic potash. When de-pigmentation is sufficient, replace the alkali with water, then dehydrate, clear in xylene, and mount in balsam or damar.

307. Neuropteroids, etc. The imagos of Ephemerida, Plecoptera, Isoptera, some Trichoptera, and the larvæ or nymphs of these as well as those of the Odonata, Neuroptera, and Mecoptera should be preserved in 80–85 per cent alcohol or 10 per cent commercial formalin. It may be necessary to wet the bodies of some of these in alcohol to get them to sink in the formalin.

Fleas, lice, Mallophaga, Physopoda, and some Diptera such as the fungus gnats, may be mounted in balsam on slides. The imagos of the dragonflies and related forms are in most cases mounted on pins.

Needham and Claassen (Plecoptera of North America, 1925) recommend preserving stone flies in 70–80 per cent alcohol. Specimens may be pinned, but in most cases they shrivel and fade and so become unsightly. Wings are mounted dry on a slide, the cover glass fastened on with gummed paper. The genitalia are boiled in caustic potash and preserved in alcohol.

For many notes relating to the technique of preserving Odonata see " The Biology of Dragonflies " by Tillyard.

308. Scale Insects. The scales should be soaked in a 5 or 10 per cent solution of caustic potash (KOH) until transparent, or boiled for 5 minutes or more in the solution. If any difficulty is experienced in getting the insects transparent, they should be removed from the potash and punctured with a fine needle. The body contents and eggs should then be pressed out and the insects transferred to a Syracuse watch glass containing a 70–80 per cent solution of glacial acetic acid. From the potash (or from the acid), transfer to distilled water where the specimens should be left for 5–15 minutes, the washing being repeated once or twice. Place in 95 per cent alcohol for 10 minutes and thence to a $\frac{1}{2}$ per cent alcoholic solution of acid-fuchsin or gentian violet for 24–48 hours. Some specimens may be left in the stain for 3 or 4 days if it is desired to study the derm pores. To demonstrate the anal plates and ring, specimens may be stained for 3–4 days in

picro-creosote (picric acid, 1 gram; beechwood creosote, 100 cc.) after dehydrating in absolute alcohol. Clear in beechwood creosote, and mount in xylene balsam. (Cf. Dietz and Morrison. The Coccidæ or Scale insects of Indiana, 1916.)

If specimens are over-stained in acid-fuchsin or gentian violet, de-stain in acid alcohol, washing in three or four changes of 95 per cent alcohol for 10 minutes, then in absolute alcohol for 10 minutes more. Clear in xylene or carbo-xylene, and mount in balsam.

Very convex forms like Leucanium may be flattened out by slitting the margin in three or four places. The caudal structure may be dissected out and separately mounted. Since traces of alkali affect the permanency of some stains, J. Howard Gage (Ent. News, 30 : 142, 1919) treats the specimens after several washings in distilled water, with an acid stain made up of acid-fuchsin, ½ gram; 10 per cent hydrochloric acid, 25 cc.; distilled water, 300 cc. Stain for 20–40 minutes, then dehydrate, clear, and mount in balsam.

309. Aphids should be mounted on slides. If the time is limited the living specimen may be put in a drop of Canada balsam on the center of the slide and immediately covered. The body fluids will at first cloud the balsam to some extent, but in time the object clears. Another short method is to kill the specimen in 95 per cent alcohol, where it should be left for 10–15 minutes; then mount directly in Euparal on the slide.

Better preparations may be made from specimens killed in hot 95 per cent alcohol. The alcohol should be heated in a water bath to prevent ignition. When the aphid has been killed it may be mounted directly in Euparal, or it may first be dehydrated in absolute alcohol, then cleared in xylene or carbo-xylene and mounted in balsam. Some workers prefer mounting in damar.

310. Lepidoptera. Hairless caterpillars may be killed in hot water and preserved in 10 per cent commercial formalin or 80 per cent alcohol. It is well to change the preserving fluid once or twice unless the quantity used is large in proportion to the volume of the larva.

Adult butterflies and moths should be pinned through the thorax. For purposes of identification, it is not necessary to spread the specimens. Directions for spreading may be found in Banks (1909), Comstock (1913), and works of a similar character.

Bleaching wings of Lepidoptera. Carefully detach the wing from a dry specimen, dip it into alcohol, and then immerse it for a moment in a 10 per cent solution of hydrochloric acid. Next soak the wing, upper side down, in a freshly made solution of Labarraque (Eau de Labarraque). Bleaching may be hastened by removing the wing from time to time from the solution, dipping it into the hydrochloric acid and replacing it in the solution. When bleached, wash it in 80 per cent alcohol and transfer it to 95 per cent alcohol. From the 95 per cent alcohol it may be cleared in carbo-xylene or from absolute alcohol in xylene. Mount in thin balsam. Tower recommends boiling the wing in hydrogen peroxide until the scales are bleached. Wash in water for 15 minutes, then for 15 minutes in 70 per cent alcohol. Stain for 1–3 hours with cyanin, gentian violet, or rosanilin; wash for 15 minutes in 50 per cent alcohol and for 30 minutes in water. Repeat, washing if necessary to clear the membrane of the stain. Mount in glycerin jelly. For balsam mounts, bleach and wash as above, stain in cyanin for 2 hours, wash in 50, 70, and 90 per cent alcohol, 10 minutes each, to remove the surplus stain, clear in clove oil and mount in xylene balsam. Bleaching may also be done with Diaphanol.

To study the venation it is sufficient in many cases to remove the scales with a stiff camel's hair or badger hair brush.

311. Staining. Different stains have been suggested for coloring the wing veins, among them a 1 per cent aqueous solution of eosin or of acid-fuchsin, or a 1 per cent aqueous or alcoholic solution of erythrosin. The time necessary to stain successfully will depend upon the wing. Eosin and erythrosin may require several days. Before staining, the wing should be soaked in distilled water. After staining, wash in alcohol, dehydrate, clear, and mount in balsam.

312. Diptera. The larvæ of Diptera may be killed in hot water, placed for a few hours in 50 per cent alcohol, then in 70 per cent, and finally in 80–85 per cent for preservation. The larvæ of the Stratiomyiidæ are covered with a calcareous deposit which must be removed before good balsam mounts can be made. To do this, drop the larva or puparium in a weak solution of hydrochloric acid. When active effervescence ceases, wash in water rendered alkaline by the addition of a little caustic potash, then in distilled water, then dehydrate, clear in xylene, and mount in balsam.

De Meijere (Tijdschrift voor Entomologie, 68 : 211, 1925) recommends killing the larvæ of Agromyzidæ in hot water or alcohol and clearing in strong carbolic acid. They may be mounted entire in Venetian turpentine directly from alcohol or from the carbolic acid. Pupæ that have become too dark may be bleached by leaving for several hours in Diaphanol, before transferring to the slide.

Larvæ may also be mounted directly, from the 95 per cent or absolute alcohol, in Euparal, or may be cleared in xylene and mounted in balsam.

The imagos of Diptera should be pinned, the smallest specimens on double mounts, the others on No. 1 or 2 pin, except for a few of the largest, which may require a No. 3 pin. The Muscoid flies should be pinned through the thorax a little to one side of the median line. Most of the nematocerous Diptera make good balsam mounts.

313. Thick-bodied larvæ do not make neat balsam mounts, nor is it possible in many cases to distinguish details of structure in them. To overcome this difficulty, the insect may be soaked for some hours in 10 per cent caustic potash until the darker parts become pale, and slit on the side. The dissolved internal tissues may then be squeezed out, and the insect thoroughly washed to remove the alkali. It may then be dehydrated, cleared, and mounted in balsam. If care is taken to support the cover glass sufficiently, the mount will not be distorted.

314. Coleoptera. The larvæ of beetles may in general be treated like dipterous larvæ. The adults should be pinned through the right elytra. Those too small for a No. 2 pin may be mounted on cardboard points.

315. Hymenoptera. The larvæ of Hymenoptera may be preserved in the same manner as the dipterous larvæ, or in the manner described for Protura. The adults should be pinned through the center of the thorax, or, in the case of the minuter forms, mounted on points.

APPENDIX

SUGGESTIONS TO THE BEGINNER ON A PROCEDURE FOR FIXING, SECTIONING, AND STAINING HISTOLOGICAL SPECIMENS — PARAFFIN METHOD

The time given for the various steps is subject to considerable variation, depending upon the size and structure of the material.

1. Anesthetize the animal, then kill it and remove a piece of the organ which is to be studied. The fragment should preferably not exceed a few millimeters in thickness. If an insect or other small arthropod is to be studied, it may be killed by being dropped into hot (60–80° C.) water or fixing fluid, and then pierced to permit better penetration of the liquid.
2. Fix for 24 hours in Bouin's, Zenker's, Gilson's or Dietrich's fluid (§§ 12, 14, 19, 20).
3. Pass through 50 and 70 per cent alcohol, treating the material that was fixed in Gilson's or Zenker's fluid with 70 per cent iodinized alcohol until the color is unaffected (§§ 12, 14).
4. Preserve in 80 per cent alcohol.

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5. Pass through 85 and 95 per cent alcohol into absolute alcohol during the course of 24 or more hours. This period may be much reduced for soft tissue or very small pieces of material.
6. Clear in xylene or chloroform for 1 or 2 hours (§§ 51, 161).
7. Infiltrate with xylene-paraffin for 1 hour (§ 52).
8. Infiltrate with paraffin (52–56° C.) for 2 hours (§ 52).
9. Imbed and cool quickly (§ 53).
10. Section with the microtome, removing the ribbon with a brush (§ 56).
11. Put albumen fixative on the slide, adding a few drops of distilled water, and arrange the sections on the slide (§§ 140, 141).
12. Warm the slide to spread the sections, then dry in air for 12 or more hours (§ 140).
13. Remove the paraffin with xylene (10–15 minutes).
14. Remove the xylene with absolute alcohol (5 minutes).
15. Pass through 95 and 60 per cent alcohol to water.
16. Stain for 5–20 minutes in Delafield's hematoxylin (§ 93).
17. Rinse for a few minutes in water.
18. Transfer to 60 per cent, then into 95 per cent alcohol.
19. Counter-stain for ½ minute (more or less) in an alcoholic solution (½ per cent) of eosin (§ 115).
20. Remove excess stain in 95 per cent alcohol.

21. Dehydrate in absolute alcohol.
22. Clear in xylene and mount in balsam (§ 162).

Should it be desired to stain with iron hematoxylin, follow the directions as above as far as No. 16, then proceed as follows:

16. Place the slide in a 4 per cent solution of iron alum (§ 95) for 2 or more hours.
17. Rinse in water.
18. Stain for 4–24 hours in iron hematoxylin (§ 95).
19. Rinse off the superfluous stain in water.
20. Place the slide in a $2\frac{1}{2}$–4 per cent solution of iron alum. From time to time, examine under a microscope until the cytoplasm appears gray and the chromatin material deep blue, then rinse in water.
21. Wash in running water for 15 minutes or more. The iron alum must be removed.
22. Transfer to 95 per cent alcohol for 1 minute.
23. Counter-stain with Congo red (§ 114) or eosin (§ 115) until the cytoplasm is sufficiently colored. A few seconds may suffice.
24. Transfer through 95 per cent alcohol to absolute.
25. Clear in xylene and mount in balsam (§ 162).

CONVERSION TABLE

LINEAR MEASURE

One micron = 1 mu (μ) = 0.001 millimeter.
One millimeter = 1 mm. = 0.001 meter = 0.03937 inch or approximately $\frac{1}{25}$ inch.
One meter = 1000 millimeters = 39.3704 inches.
One inch = 25.399772 mm. = approximately 25.4 mm.

CUBIC MEASURE

One cubic centimeter = 1 cc. = 0.001 liter.
One liter = 1000 cubic centimeters = 2.11 pints = approximately 1 quart.
One fluid ounce = 29.578 cc. or approximately 30 cc.

WEIGHT

One fluid ounce = 29.578 cc.
One apothecary's ounce = 480 grains = 31.103 grams.
One avoirdupois ounce = 437.5 grains = 28.349 grams.
One avoirdupois pound = 453.59 grams.
One gram = 15.432 grains = 1 cubic centimeter of water at maximum density.
One kilogram = 1 kilo = 1000 grams = 2.204 avoirdupois pounds.

TEMPERATURE

To convert Fahrenheit to Centigrade, subtract 32 from the Fahrenheit reading and multiply the remainder by $\frac{5}{9}$. Example: 98° F. = $(98 - 32)\frac{5}{9}$ = 36.66° C.

To convert Centigrade to Fahrenheit, multiply the Centigrade reading by $\frac{9}{5}$ and add 32. Example: 50° C. = $(50)\frac{9}{5} + 32$ = 122° F.

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